Effects of Solvent Engineering and Chemical Modification on the Activity and Stability of Wheat β-Amylase

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2014

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Abbreviations

AU	arbitrary unit
BA	β-amylase
BacBA	*Bacillus* β-amylase
BBA	barley β-amylase
βME	β-mercaptoethanol
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
EG	ethylene glycol
EI	enzyme-inhibitor complex
GA	glutaraldehyde
K_a	association constant
k_{cat}	molecular activity
K_m	Michaelis constant
mPEG	2,4-bis (*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine
p*I*	isoelectric point
SBA	soybean β-amylase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TNBS	2,4,6-trinitrobenzenesulfonic acid
V_{max}	maximum velocity
WBA	wheat β-amylase

Introduction

β-Amylase (BA) [EC 3.2.1.2] or α-1,4-glucan maltohydrolase is an exo-enzyme that catalyzes the cleavage of α-1,4-glucosidic bonds of polysaccharides and liberates maltose from the non-reducing ends. It is a member of family 14 of the sequence-based classification of glycoside hydrolases (*1*). BAs are found in higher plants and certain bacteria, and there are some differences between bacterial and plant BAs in their ability to bind and hydrolyze raw starch (*2*). This binding ability is credited to a starch-binding domain located at the C-terminus of the sequence (*3*).

Enzymes can easily be denatured by a slight change of the environmental conditions such as temperature, pressure, pH, and ionic strength (*4*). However, stabilization of enzymes can be achieved in several ways: screening for more stable ones (from thermophiles and extremophiles), chemical modification, site-directed mutagenesis, immobilization, and solvent engineering or modifying the enzyme reaction conditions with stabilizing additives (*5*-*10*).

Unlike soybean, barley, and sweet potato, wheat β-amylase (WBA) is not a common source of BA for starch-saccharification industries so far. However, the sources of BA supply have changed due to escalating prices of the major sources of BA. WBA is prepared from wheat bran, which is an industrial by-product in the production of wheat starch and gluten. It is a cheap alternative source of BA for industries. Nevertheless, it is relatively thermo-labile as compared with BAs of other crops and microbes.

Temperature has substantial effect on the molecular activity as well as

conformation of enzymes. The inhibitor constant (K_i) values of inhibitors were affected by changes in temperature (*11, 12*). pH alters the ionization of the functional groups and conformation of enzymes and hence, may affect substrate or inhibitor binding. The inhibition type by glucose, maltose, and cyclohexa-amylose on soybean β-amylase (SBA) was pH dependent (*13*).

The fluorescence change of enzymes can be a good probe for examining the binding of substrates or inhibitors (*14*). A small change in the catalytic residue of subtilisin (Ser221 to Cys221) led to considerable decrease in its binding affinity to *Streptomyces* subtilisin inhibitor (*15*). The changes in the states of tryptophan and tyrosine residues of an enzyme may be associated with its activity.

Chemical modification is one of the techniques used in stabilizing enzymes, and various chemicals were identified that modify specific residues of enzymes and confer better stability. Among which, 2,4,6-trinitrobenzenesulfonic acid (TNBS), 2,4-bis (*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine (mPEG), and glutaraldehyde (GA) are known to make covalent interaction with the amino groups of enzymes (*16-19*). Stabilization and activation of WBA has practical applications for its use in food, bio-ethanol, and starch-saccharifying industries. In Chapter 1, the effects of various additives on the thermal stability and activity of WBA were evaluated through solvent engineering. In Chapter 2, the inhibitory interactions of maltose, glucose, and related carbohydrates with WBA; and the modes of inhibition by maltose and glucose, kinetics, and thermodynamic properties were examined. In Chapter 3, the interaction of WBA with maltose and glucose on the fluorescence intensity of WBA was evaluated. In Chapter 4, the effects of chemical modification of the amino groups of WBA by TNBS, mPEG and GA, on its thermal stability and activity were studied.

Chapter 1

Characterization and Solvent Engineering of Wheat β-Amylase for Enhancing Its Activity and Stability

Introduction

BA hydrolyses the α-1,4-glucan bonds in amylosaccharide chains from the non-reducing ends and generates maltose. It has considerable industrial applications together with starch debranching enzymes and α-amylases. However, the well-characterized BAs are neither active nor stable at higher temperatures > 65^{o}C (*20*). In many findings, α-amylase is more stable compared to BA of the same origin (*4, 21, 22*). Different techniques can be used to improve the thermal stability and activity of enzymes. Various aspects of the techniques are considered to choose the proper ones. For instance, site-directed mutagenesis cannot be used in enzymes that are used in food industries. In solvent engineering, selection of appropriate additives is dependent on the nature of the enzyme and there are no established rules to select effective additives for improving enzyme functions (*23*). The thermostabily of BAs has been substantially enhanced by modifying the solvent with additives (*23, 24*).

In the hydrolysis of various polysaccharides, different amylases give oligosaccharides of specific length as end products. For this reason, amylases with unique properties need to be studied for their applications in various industries like in starch-saccharification, production of bio-ethanol, and food (*25*). The T_{opt} of

Clostridium thermosulphurogenes BA is 75°C (*20*), T_{50} of barley β-amylase (BBA) is 56.8°C, and that of SBA is 63°C (*26*).

Therefore, enhancing the activity and thermostability of WBA has good prospect for starch-saccharification industries. In this study, we used a commercially-available WBA preparation, Himaltosin, without further purification because it is already purified from other protein contaminants and utilized in industries. In this paper, we describe the kinetic and thermodynamic properties of WBA and improvement of its activity and thermostability via solvent engineering using various additives.

Materials and Methods

Materials - Himaltosin GS (Lot 2S24A), a commercial preparation of WBA, was purchased from HBI Enzymes (Osaka, Japan). This preparation was filtered with a Millipore membrane filter (Type HA; pore size: 0.45 μm) and used without further purification. According to the manufacturer, the Himaltosin preparation contains 90% starch as a stabilizer, and all of the protein is BA and α-amylase was not detected. Himaltosin was suspended in 20 mM sodium acetate buffer (pH 5.4) at 25°C to be 0.3 mg/ml. In this paper, this buffer is hereinafter referred to as buffer A. The WBA protein content was expected to be 0.03 mg/ml in the suspension and it was followed by filtration with Millipore membrane filter. However, the protein concentration in the filtrate was less than 10% of the expected content, suggesting that > 90% of the WBA protein was remained with starch on the filter. The WBA concentration was determined spectrophotometrically in buffer A using the absorbance (*A*) of 1.40 ± 0.02 at 281 nm

with a 1.0 cm light-path for the WBA solution at the concentration of 1.0 mg/ml (*27*). The molecular mass of 57.7 kDa for WBA was used to determine the molar concentration of WBA. Under the standard condition in this study, the concentration of WBA in the enzyme-reaction solution was set to 15-30 nM. The starch concentration due to the stabilizer starch (0.027%, w/v) was completely removed by filtration with Millipore membrane filter. Soluble starch (Lot M7H1482) as substrate and maltose (Lot M1F7568) as standard for the activity assay were obtained from Nacalai Tesque (Kyoto, Japan). The substrate has a weight-average molecular weight of 1.0×10^6 according to the manufacturer, and thus the average degree of polymerization of glucose unit is estimated to be 6,000. Neocuproine-HCl (2,9-dimethyl-1,10-phenanthroline, Lot 032K2533) as coloring reagent B in the neocuproine method was from Sigma (St. Louis, MO, USA). Coloring reagent A (0.38 M Na_2CO_3, 1.8 mM $CuSO_4$, and 0.2 M glycine) in the neocuproine method and all other chemicals were purchased from Nacalai Tesque. All enzyme reactions were carried out in buffer A, pH 5.4.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) - SDS-PAGE was performed in a 10% polyacrylamide gel under reducing conditions (*28*). The Himaltosin preparation was suspended in buffer A to the concentrations of 1, 2, and 5% (w/v). The solution was filtered with a Millipore membrane filter (Type HA) and reduced by treatment with 10 mM dithiothreitol (DTT). The solution was applied to SDS-PAGE with a constant voltage of 150 V for 80 min. The proteins were stained with Coomassie Brilliant Blue R-250. The molecular-mass marker kit consisting of rabbit muscle phosphorylase *b* (97.2 kDa), bovine serum albumin (66.4 kDa), hen egg albumin (44.3 kDa), and hen egg white lysozyme (14.4 kDa) was a product of Takara Bio (Otsu,

Japan).

Measurement of enzyme activity - Various initial concentrations of the soluble starch substrate [0, 0.09, 0.45, 0.90, 1.13, 1.35, 1.80, 2.02, 2.25, 2.70, 2.93, and 3.15% (w/v)] in the reaction solution were prepared in buffer A at 25^{o}C. The WBA solution in the same buffer was filtered with a Millipore membrane filter (Type HA) and kept in ice water for immediate use. The various concentrations of starch were hydrolyzed by WBA (30 nM) for 0, 2.5, 5.0, 7.5, and 10.0 min at 25^{o}C. The reaction was stopped by adding 300 μl of 0.1 M NaOH into 100 μl of the enzyme-reaction solution. The amount of the reducing sugar produced in the enzyme-reaction solution was determined by the neocuploine method as follows (*11*). Reagent A and reagent B, 250 μl each, were mixed with 50 μl of the enzyme-reaction solution, boiled for 8 min, and diluted with 550 μl of water after cooling in ice water. The activity was measured at 450 nm using a Beckman-Coulter DU 800 spectrophotometer (Batavia, IL, USA) (*29, 30*). The enzyme activity was determined by measuring the velocity (v) of reducing sugar production, and the reaction velocity was analyzed by the Michaelis-Menten kinetics. The maximum velocity (V_{max}) and Michaelis constant (K_m) were obtained from the v *vs.* the substrate concentration ($[S]_o$) plots using KaleidaGraph 4.1 (Synergy Software, Reading, PA, USA). The molecular weight of WBA is 57,500 (*31*) and was used for the evaluation of the molecular activity (k_{cat}).

The optimum temperature - Starch solution (900 μl) in buffer A was mixed with the WBA solution (0.15 μM; 100 μl) in the same buffer for 0, 2.5, 5.0, 7.5, and 10.0 min at 25^{o}C after incubation at 15, 25, 35, 45, 55, 65, and 75^{o}C in a water bath for 10 min. The

initial concentrations of starch and WBA in the reaction solution were 1.8% and 15.0 nM, respectively. The reaction was stopped by adding 300 μl of 0.1 M NaOH into the enzyme-reaction solution (100 μl). Then the enzyme activity was determined by the neocuproine method. The optimal reaction temperature at which WBA exhibited the highest activity was referred to as the T_{opt}.

Thermal inactivation of WBA - The substrate and WBA solutions were prepared in buffer A. The enzyme solution was incubated at 25, 35, 45, 55, and 65^{o}C for 10, 20, and 30 min and cooled at 25^{o}C for 3 min in a water bath. The substrate solution (2%, w/v; 900 μl) was mixed with the heat-treated WBA solution (0.3 μM; 100 μl) at 25^{o}C and incubated for 0, 2.5, 5.0, 7.5, and 10.0 min. The initial concentrations of substrate and enzyme in the reaction solution were 1.8% (w/w) and 30 nM, respectively. The activity was assayed as aforementioned by the neocuproine method and the first-order rate constant k of the thermal inactivation was determined assuming pseudo-first order kinetics by plotting ln (v/v_o) against the heat-treatment time (t) (Eq. 1), where v is the initial velocity of the enzyme with heat treatment at each incubation temperature and v_o is that obtained without heat treatment and at 25^{o}C. The activation energy E_a of the thermal inactivation was obtained from the Arrhenius plot (Eq. 2), and the standard Gibbs energy difference of activation for thermal inactivation ($\Delta G^{o\ddagger}$), the standard enthalpy difference of activation ($\Delta H^{o\ddagger}$), and the standard entropy difference of activation ($\Delta S^{o\ddagger}$) were obtained from the Eyring plot according to Eqs. 3 and 4 (*11*, *32*).

$$\ln (v/v_o) = -kt \quad (1)$$

$$\ln k = -(E_a/R)(1/T) \quad (2)$$

$$\Delta G^{o\ddagger} = -RT[\ln hk/ k_B T] \quad (3)$$

$$\ln (hk/ k_B T) = (\Delta H^{o\ddagger}/RT) + (\Delta S^{o\ddagger}/R) \qquad (4)$$

where k_B, h, and R are the Boltzmann, Planck, and gas constants, respectively. T is temperature in Kelvin.

Activation and thermostablization of WBA using additives - The WBA solution in buffer A was mixed and incubated with equal volume of various additives in the same buffer at 25, 45, 55, and 65°C in a water bath for 30 min before hydrolyzing soluble starch. The initial concentrations of WBA and substrate in the reaction solution were 30.0 nM and 1.8%, respectively. The additive concentrations in the reaction solution were: 45.5, 91, 182, and 364 mM glucose, NaCl, and glycine; 45.5 and 91 mM L-arginine; 0.9, 1.8, 3.6, and 45.5 mM of L-aspartate; 45.5 and 91 mM L-cysteine and glutathione (GSH); 0.18 and 0.45% (w/w) gelatin; 0.91, 1.82, and 5.5% (w/w) ethanol and 2-methyl-2-butanol (2M2B); 0.45 and 2.7% (w/w) dimethyl sulfoxide **(**DMSO) and dimethylformamide (DMF); and 0.91 and 5.5% glycerol, ethylene glycol (EG), and β-mercaptoethanol (βME). Their effects on activation and thermal stabilization of WBA were examined. The enzyme-additive mixture solution (0.2 ml) was diluted with water (0.55 ml), and the enzyme activity was measured by the neocuproine method.

Results

Kinetic parameters of WBA - The WBA preparation, Himaltosin, showed a single band in SDS-PAGE with molecular mass of 57.7 kDa (Fig. 1). This value is in good agreement with those (54.0-64.2 kDa) so far reported (*27, 31*). In this paper, we used

57.7 kDa for the molecular mass of WBA. This indicates that the Himaltosin preparation is composed of solely BA as a protein component, although it contains 90% (w/w) starch as a stabilizer. There was no starch carried over from the stabilizer starch into the reaction solution as examined by the starch-iodine reaction. Therefore, the enzyme preparation was treated as WBA without further purification.

The initial velocity (v) of the WBA-catalyzed hydrolysis of different concentrations of soluble starch was examined in buffer A at 25^{o}C at the enzyme concentration of 30 nM. The dependence of v on substrate concentration exhibited Michaelis-Menten profile (Fig. 2). The K_m, V_{max}, and k_{cat} were determined to be 1.0 ± 0.1 % (w/v), 2.8 ± 0.1 μM s^{-1}, and 94 ± 3 s^{-1}, respectively, by fitting the experimental data to the Michaelis-Menten equation.

Thermal inactivation of WBA - WBA at the concentration of 0.3 μM was treated thermally as described in the Material and Methods section, and the WBA concentration in the enzyme-reaction solution was fixed to 30 nM. The enzyme activity decreased with the progress of the heat treatment at every temperature examined (Fig. 3). The enzyme activity in the hydrolysis of soluble starch was evaluated by measuring the initial velocity (v) in the same buffer at 25^{o}C. The v value measured at the zero min incubation at each temperature was designated as v_o. The relative activity (v/v_o) observed after incubation at various temperature decreased progressively with increasing the incubation time. The semi-log plots of v/v_o against incubation time showed linear relationship at the respective incubation temperatures (Fig. 3), indicating that the thermal inactivation process of WBA follows the first-order kinetics. The first-order rate constant (k) at the indicated incubation temperature was evaluated from

the slope of the plot.

The activation energy E_a value of the thermal inactivation of WBA in buffer A was 36 ± 1 kJ mol^{-1} from the slope of the Arrhenius plot (Fig. 4). The $\Delta G^{o\ddagger}$, $\Delta H^{o\ddagger}$, and $T\Delta S^{o\ddagger}$ values for the thermal inactivation were found to be 92 ± 1, 33 ± 1, and -59 ± 1 kJ mol^{-1} respectively, at 25^{o}C from the slope of the Eyring plot (Fig. 5). As E_a is defined theoretically as $\Delta H^{o\ddagger} + RT$, the $\Delta H^{o\ddagger}$ value is calculated to be 34 ± 1 kJ mol^{-1}, which is in good agreement with the value obtained from the Eyring plot.

The optimum temperature of WBA - WBA activity was measured at various reaction temperatures (Fig. 6). The maximal activity in starch hydrolysis was obtained at 55^{o}C, being the optimum temperature (T_{opt}) of this enzyme in buffer A, pH 5.4.

The T_{50} of WBA - The heat inactivation of WBA was studied by incubating the enzyme at various temperatures for 30 min before hydrolyzing the substrate. The enzyme activity was observed to decline with heat treatment even at moderate temperatures (45 and 55^{o}C). The T_{50} of WBA, which is the temperature at which the enzyme loses half of its activity with thermal treatment for 30 min was determined to be 50 ± 1^{o}C (Fig. 7) by plotting the residual activities (%) against temperature of incubation. WBA has lost 30% of its activity at 45^{o}C, 76% at 55^{o}C, and 95% at 65^{o}C after 30 min of incubation.

Effect of additives on WBA activity - The catalytic activity of WBA in starch hydrolysis was examined in the presence of various additives at 25^{o}C without heat treatment. Majority of the evaluated additives did not enhance the enzyme activity (Fig.

8). However, it was noted that ethanol at the concentration of 0.91-5.50%, w/w (or 0.2-1.2 M) enhanced the activity by 24% and low concentration (1.8%) of DMF by 11%. On the other hand, the activity was not much affected with the addition of NaCl and glycine up to 364 mM; and DMF, DMSO, and EG up to 5.5%. The addition of cysteine up to 91 mM and aspartate up to 45.5 mM showed no substantial effect on the activity, and gelatin (0.18-0.45%, w/w) had no effect either. The other additives inhibited the activity to varying degrees. Especially, the activity was reduced to almost 10% with the addition of 0.91-5.5%, w/w (or 0.11-0.71 M) βME; and to zero with 45.5-91 mM arginine. The activity decreased with increasing glucose and GSH concentrations, and 50% of the activity was lost with 182 mM glucose, and 75% with 91 mM GSH.

Effect of additives on the thermostabilty of WBA - The rate of starch hydrolysis by heat treated WBA was examined in solvent modified by various additives. Glycine was the best in improving the thermal stability of WBA followed by gelatin. The T_{50} of WBA was improved by 6^{o}C with 182 mM glycine and by 4^{o}C with 0.18% (w/w) gelatin (Fig. 9). The half-life of WBA was enhanced by 25 min and 17 min with glycine and gelatin, respectively, at 55^{o}C (Table 1). It should be noted that the residual activities after thermal treatment at 45^{o}C was 97% and 92% in the presence of 0.18% (w/w) gelatin and 182 mM glycine although it was only 75% in the absence of additives. Aspartate (45.5 mM) and DMSO (5.5%, w/w) had no effect on the stability of WBA. βME (0.91%), ethanol (0.91%), NaCl (182 mM), and arginine (45.5 mM) decreased the stability of WBA, and the T_{50} values were 46-47^{o}C. Glucose (182 mM), cysteine (46 mM), and GSH (91 mM) decreased the stability with the T_{50} values of 35-45^{o}C.

Discussion

In general, depending on the nature and concentration of the additives, they affect the protein conformation in: (a) screening effect, where the electrostatic repulsion between similarly charged groups of proteins is reduced by cosolvent ions; (b) solvophobic effect, where ion pair formation occurs, favoring protein folding; and (c) modification of water structure leading to hydrophobic interactions in proteins (*21, 33*). The catalytic activity and thermostability of various enzymes were considerably enhanced through solvent engineering (*10, 23, 24, 34, 35*).

The K_m (1.0 ± 0.1%, w/v) and k_{cat} (94 ± 3 s^{-1}) of WBA were evaluated at pH 5.4 and at 25^oC using soluble starch as a substrate (Fig. 2). Similarly, the K_m of glutenin-adsorbed WBA was reported to be 0.15% (w/v) using soluble starch substrate (*36*), indicating that WBA (which is free in the reaction solution) used in our study has lower affinity to soluble starch as compared with WBA adsorbed on glutenin. The other available reports on the kinetic parameters of WBA were using maltotriose, maltoheptaose, amylodextrin, and amylopectin substrates under various reaction conditions (*31, 37*). The K_m values of plant BA have been reported to be in the range of 0.2-0.7% (w/v) for soluble starch. The value obtained in this study is apparently higher than those. However, the kinetic parameters for WBA and other plant BA have not been examined under the same reaction conditions using the same soluble starch preparation. Therefore, it is not suitable to discuss the enzyme functions by comparing the kinetic parameters so far reported. In this paper, we have reported the kinetic parameters (K_m

and k_{cat}) of WBA obtained under the fixed conditions with the WBA and soluble starch preparations in the same lot. It should be noted that the activity and stability of WBA are affected easily by additives in the reaction solution. This means that the kinetic parameters of WBA must be evaluated with the data collected from the experiments conducted carefully under the same conditions.

Thermodynamic parameters for the heat inactivation of WBA showed that the $T\Delta S^{o\ddagger}$ value (-59 ± 1 kJ mol^{-1}) was greater in magnitude than the $\Delta H^{o\ddagger}$ value (33 ± 1 kJ mol^{-1}) and thus, it can be inferred that the heat inactivation of the enzyme is entropy-driven. The T_{opt} of WBA is 55^{o}C (Fig. 6). It has been reported that the T_{opt} of *Sorghum bicolor cv* BA is 50^{o}C (*22, 25, 38*) while α-amylase from the same cereal crop has T_{opt} of 70^{o}C corresponding with the fact that BAs in most of the cases are lower in thermostability than their respective α-amylases of the same origin (*21, 22*). The T_{50} of WBA was determined to be 50 ± 1^{o}C (Fig. 7), which is lower than those of SBA, 63.2^{o}C, and of BBA, 56.8^{o}C (*26*).

In the present study, we have examined the effects of additives on the kinetic parameters and thermostability of WBA. The additives are supposed to have effects on the structures of WBA, soluble starch substrate, and bulk water. For example, when we observed decrease in activity by the addition of an additive, there might be some reasons considered such as inhibition of the enzyme by the additive, conformational changes of the enzyme and/or starch substrate by the additive, etc. Therefore, strict interpretation of the molecular effects of the additives on the activity and stability of WBA seems to be difficult, and further studies are needed. Thus, in this paper we tried to present the effects of the additives without describing the molecular-mechanistic insights of the cause for the effects, although some possible comments have been made

with references to other enzymes. The molecular-mechanistic study for the effects of the representative additives is required.

Ethanol enhanced the activity of WBA by up to 24%, and low concentration of DMF slightly enhanced it (Fig. 8). The activation of acetylcholinesterase (AchE) by ethanol was reported and explained as that it non-competitively alters the hydrophobic-interaction site and subsequently induces favorable conformation to the active site (*39*). However, higher concentration of ethanol had a destabilization effect on WBA (Fig. 9B). This agrees with a finding that, high concentration of ethanol (> 800 mM) can perturb the structure of water around hydrophilic area of AchE causing instability to the conformation of the enzyme (*39*). This might be the case for the effects of ethanol on WBA. These effects should be considered also from the viewpoint of solvent polarity. The effects of various alcohols on the enzyme structure and activity have been extensively studied with thermolysin, a thermophilic and halophilic metalloproteinase produced by *Bacillus thermoproteolyticus*. Thermolysin is remarkably activated and stabilized by neutral salts such as NaCl, and is inhibited by increasing concentration of alcohols and the degree of inhibition is dependent on the size of alcohols (*40*). Alcohol such as 2-methyl-1-propanol (2MP), which binds tightly to the active site inhibits thermolysin strongly, whereas alcohols with the sizes larger or smaller than 2MP bind weakly to the active site and inhibit thermolysin weakly (*41*). This suggests that enzyme activity could be controlled intentionally using additives with suitable sizes. These lines of evidences provide information for the optimal conditions for thermolysin-catalyzed synthesis of a precursor of the sweetener, aspartame (*7*). As shown with thermolysin to find the optimal conditions for its inhibition by alcohols, it should be possible to find the optimal conditions for industrial application of WBA by

examining the effects of alcohols on the activity and stability of WBA by systematically changing the size of alcohols.

Contrary to the effect of ethanol, the T_{50} of WBA is slightly improved by polyols, indicating that polyols are WBA stabilizer. Our finding agrees with reports that solvent modification by sugars and polyols had marked thermal stabilization in various enzymes (*4, 34*). Polyols were explained to preferentially be excluded from the surface layer of the protein and form a water shell around the protein, so that the protein is preserved and the conformation becomes more rigid or stable (*4, 34*). Similar mechanism of stabilization by polyols was also reported for organic polar solvents (DMSO and DMF) (*35*), and polar organic solvents have conferred a slight thermostabilization to WBA. However, sugars, which are classified also as polyols, interestingly had no stabilizing effect on WBA in our study.

The activation and thermostabilization of WBA were tested using different additives with various values of empirical parameters of solvent polarity, $E_T(30)$ (*42*) In particular, ethanol ($E_T(30)$ = 217 kJ mol^{-1}), DMSO (189 kJ mol^{-1}), DMF (183 kJ mol^{-1}), glycerol (238 kJ mol^{-1}), EG (235 kJ mol^{-1}), βME (224 kJ mol^{-1}), and 2M2B (172 kJ mol^{-1}) were examined at different temperatures. According to the results, the half-life time of WBA was enhanced at 55^{o}C with ethanol. It is suggested that thermostabilization by additives could be induced by reducing the degree of water-solvation or deformation of a water shell around the protein. However, a solvent 2M2B with a low $E_T(30)$ value decreased the activity and stability of WBA (Figs. 8B and 9B), suggesting that the effects of additives on the enzyme activity and stability are complicated and that the effect on the water-solvation might not be the main one (*43*).

Glycine was the most favorable among the evaluated additives in stabilizing WBA

followed by gelatin (Table 1) and the stabilization effects of various additive concentrations were presented in Fig. 9. It should be noted that the thermostability of WBA in the presence of favorable additives such as glycine and gelatin is almost comparable with that of SBA, which is widely used in starch-saccharification industry. This suggests that WBA could be applied more widely in food and bio-ethanol industries with employing suitable additives. A good empirical correlation between the relative stabilizing effects of glycine with the change in solvent-accessible hydrophobic surface area of the folded protein was reported (*44*). The possible protective effect of gelatin on penicillinase was stated that it combines with enzyme and form a thermostable complex (*45*).

Arginine was found to destabilize WBA in our study. Similarly, chloroperoxidase was confirmed to be inactivated by arginine mainly by the binding of a guanidinium group with the catalytic site (*46*). This inactivation effect might be the same as that given by the denaturant, guanidine hydrochloride namely, arginine might cleave the hydrogen bonds in the protein structure and increase the solubility of hydrophobic residues of the protein. On the other hand, arginine is known to work effectively in recovering human matrix metalloproteinase 7 from inclusion bodies (*47*). This suggests that arginine promotes unfolding the misfolded protein structure to lead it into the correctly folded form. While arginine seems to show multiple effects on protein stability depending on target proteins, it can be a good tool to increase the stability of WBA by selecting suitable conditions.

We tried to modify thiol groups of WBA using reducing agents like βME and GSH, whereas they destabilized WBA. This is because they attack disulfide bonds and expose proteins to heat denaturation and, hence reduces their thermostability (*48*). Probable

involvement of thiol groups in the catalytic activity of amylases has been suggested, although it is not known currently with WBA. The effects of βME and GSH should be considered in the future.

Various concentrations of NaCl exhibited destabilizing effect on WBA in our study. The following three possible reasons were suggested for salt-induced inactivation or destabilization effect on enzymes: (a) break weak hydrogen-bonds and disrupt the protein conformation, (b) attract water molecules and the enzyme coagulates by protein-protein hydrophobic interaction, and (c) high concentration of salt makes the enzyme more likely to bind with the salt ions instead of the substrate by electrostatic interaction (*49*). However, high concentration of neutral salts remarkably improves thermolysin activity (*7, 8*). The catalytic activity of thermolysin is enhanced by 4 times with the substitution of the active-site zinc with cobalt and further exponentially by NaCl up to 13-15 times (*33*).

It is well known that enzyme activity is controlled by the factors of enzyme structure and reaction environment. The structural factors and environmental factors are sometimes independent and sometimes closely connected. With their optimal combination, the optimal catalytic activity may be realized. Protein engineering (namely, site-directed mutagenesis and chemical modification) is a tool for changing the enzyme structure in a predictable and precise manner to effect a change on the catalytic process. Since the enzyme is even improved in only one side of a reaction, any changes in the rest of the reaction may also alter the catalytic process (*50*). Solvent engineering is a powerful tool in rational control of enzyme activity. In reality, both approaches are still somewhat difficult to confirm their effects, whereas they have been used successfully to alter the protein properties (*51*). At last, it should be reminded that the

protein-engineered enzymes are strictly prohibited for use in food processing in many countries in order to avoid unpredictable harms. Thus, solvent engineering must be an inevitable alternative to find the optimal conditions from the viewpoints of enhancing enzyme catalytic efficiency, guaranteeing safeness of the products, reducing the costs of production, etc. When the enzyme activity for its industrial application is increased by solvent engineering, the cost of the additive should be considered in the total cost, although it is generally much cheaper than that of the enzyme, and the improved enzyme activity and stability by the additive should decrease the enzyme amount needed and thus shortened the reaction time, which results in decreasing the running costs, utility, labor, etc.

In conclusion, it is clear that WBA is not stable at temperatures higher than 55^{o}C from the thermodynamic parameters evaluated in this study. The thermal stability of WBA was improved by glycine and gelatin possibly through convening conformation of the enzyme and reducing the interaction of the protein with the solvent. Polyols and organic polar solvents (DMSO and DMF) also conferred slight stability to the enzyme while some evaluated additives have exhibited destabilizing effect. The thermodynamic parameters indicate that WBA is thermo-labile and sufficient stabilization was achieved by solvent engineering with additives and that the heat inactivation of WBA is entropy-driven. On the other hand, it was shown that the activity of WBA activity was enhanced by ethanol and DMF probably by altering the hydrophobic interaction and inducing favorable conformation to its active center. It is suggested that WBA would be applicable to a wide range of saccharification industries such as food and bio-ethanol production with employing suitable additives.

Table 1. Effects of selected additive concentrations on thermal stabilization of WBA after incubation at 55°C with the additives for 30 min prior to starch hydrolysis.

Additives	T_{50} (°C)	k (min^{-1})	$t_{1/2}$ (min)	$\Delta G^{o\ddagger}$ (kJ mol^{-1})
none	50 ± 1	0.030 ± 0.004	23 ± 2	90 ± 1
0.18% gelatin	54 ± 1	0.017 ± 0.003	40 ± 1	92 ± 1
0.45% gelatin	54 ± 1	0.018 ± 0.001	39 ± 2	92 ± 1
91 mM glycine	54 ± 1	0.017 ± 0.003	41 ± 1	92 ± 1
182 mM glycine	56 ± 2	0.014 ± 0.006	48 ± 3	92 ± 1

The $t_{1/2}$ is the time at which WBA loses half of its activity at 55°C, and $\Delta G^{o\ddagger}$ is the standard Gibbs energy difference of thermal inactivation.

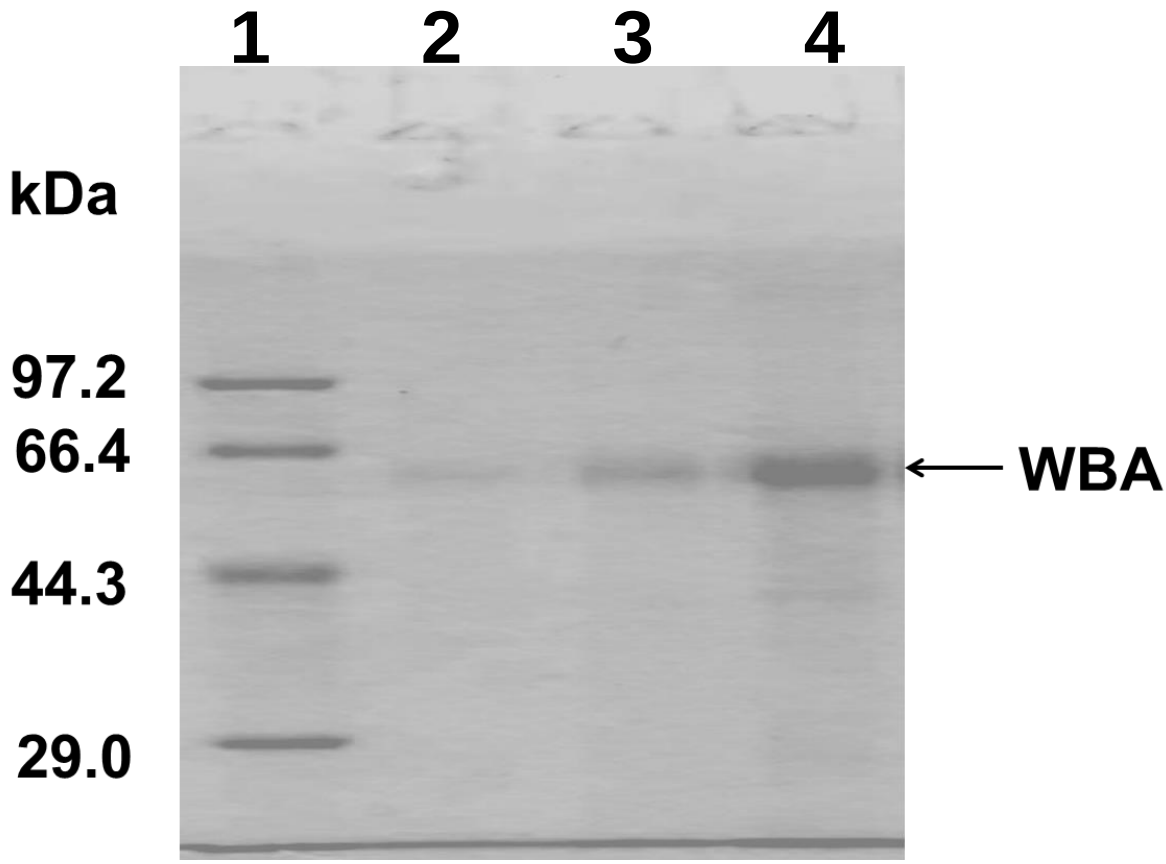

Fig. 1. SDS-PAGE of Himaltosin preparation. Molecular marker proteins (lane 1), Himaltosin preparation (w/v): 1% (lane 2), 2% (lane 3), and 5% (lane 4). The experimental conditions were given in the text (Materials and Methods).

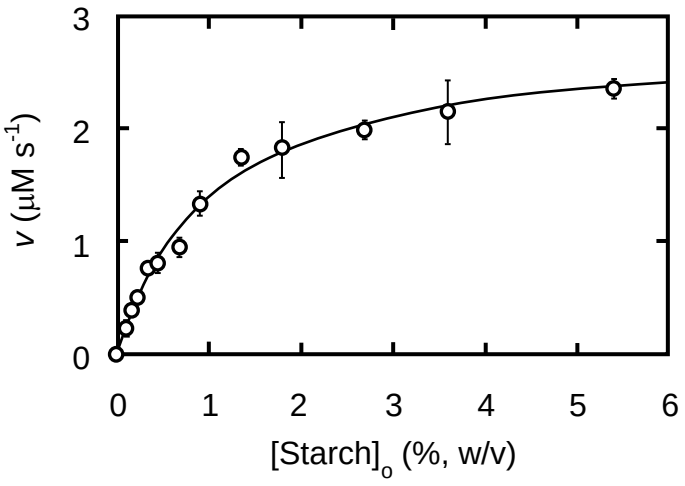

Fig. 2. Dependence of WBA activity on substrate concentration. The substrate is soluble starch**.** The hydrolysis was carried out in 20 mM sodium acetate buffer, pH 5.4, at 25^{o}C. The experiment was undertaken in triplicate.

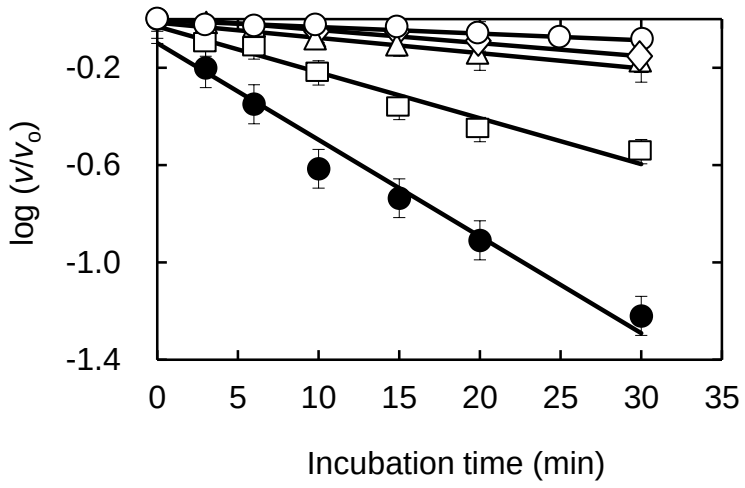

Fig. 3. Progress of the decrease in WBA activity with thermal treatment duration. WBA (0.3 μM) was treated at 25, 35, 45, 55, and 65°C for 0-30 min in buffer A, pH 5.4. WBA activity in the hydrolysis of soluble starch was evaluated by measuring the initial velocity (v) in the same buffer at 25°C. The WBA concentration in the enzyme-reaction solution was 30.0 nM. The v value measured at 0 min incubation at each temperature was designated as v_o. The logarithm of the relative activity [log (v/v_o)] was plotted against the thermal-treatment time. The markers show temperature (°C): 25, ○; 35, ◊; 45, Δ; 55, □; and 65, ●. From these semi-log plots, the first-order rate constant for the thermal inactivation of WBA was evaluated at the specified temperature of the thermal treatment.

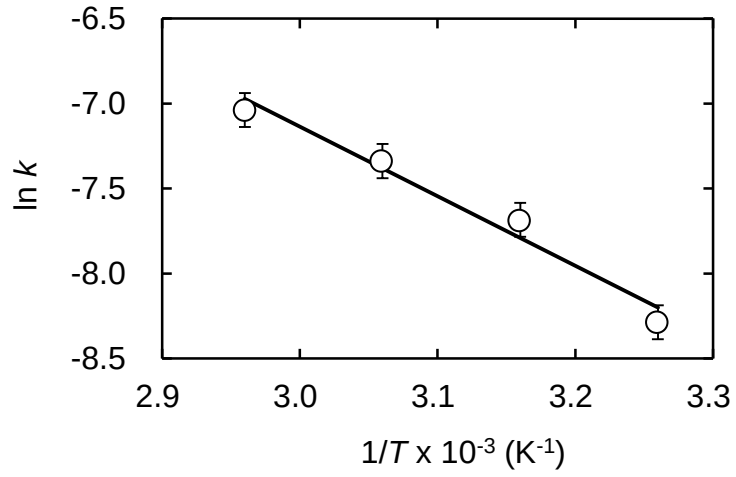

Fig. 4. Arrhenius plot of WBA for the *k* of thermal inactivation. The rate constants were evaluated from the semi-log plots as shown in Fig. 3 obtained by thermal treatment of the enzyme at various temperatures. The reaction conditions were given in the Materials and Methods.

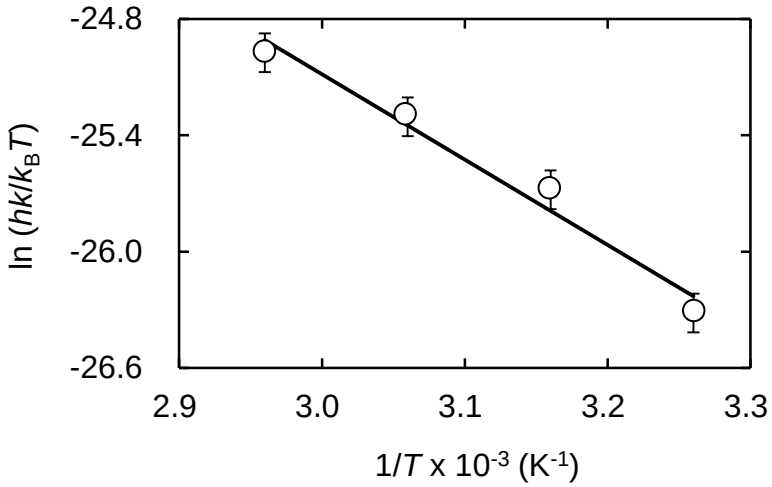

Fig. 5. Eyring plot of WBA for the *k* of thermal inactivation. The rate constants were obtained as described in Fig. 3 at each temperature. The thermodynamic properties of WBA thermal inactivation were obtained from this plot.

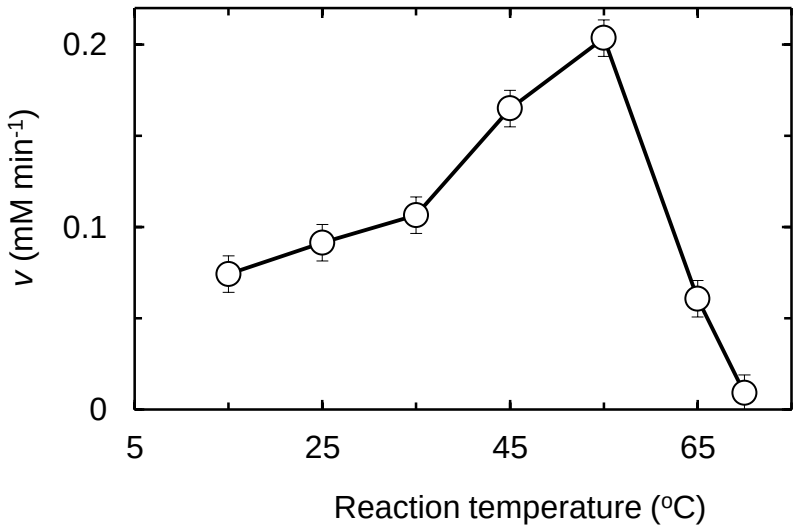

Fig. 6. Effect of temperature on the initial velocity (*v*) of WBA in hydrolyzing soluble starch. The enzyme reaction was carried out at the temperature indicated. The initial concentrations of starch and WBA in the reaction solution were 1.80% (w/v) and 15.0 nM, respectively. The optimal temperature T_{opt} was determined to be 55^{o}C.

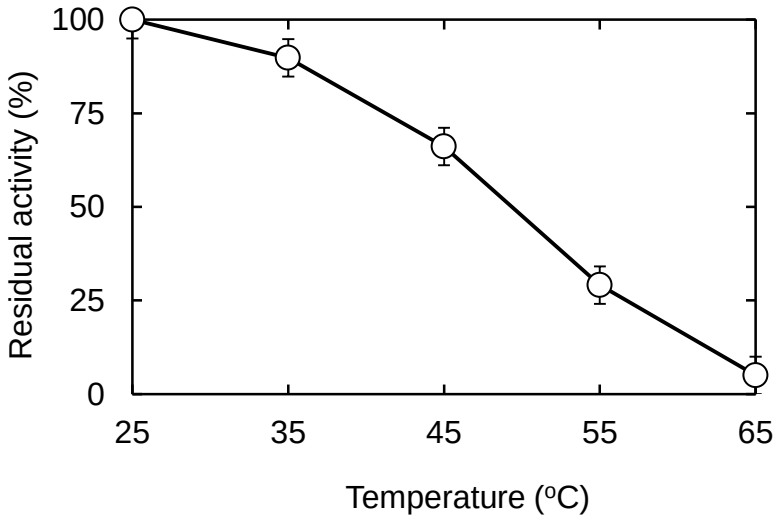

Fig. 7. Effect of thermal inactivation of WBA on the enzyme activity. The activity was assayed in buffer A (pH 5.4) in hydrolyzing starch after incubation at various temperatures indicated for 30 min. The reaction conditions are given in the Materials and Methods. The T_{50} was determined to be 50 ± 1^{o}C.

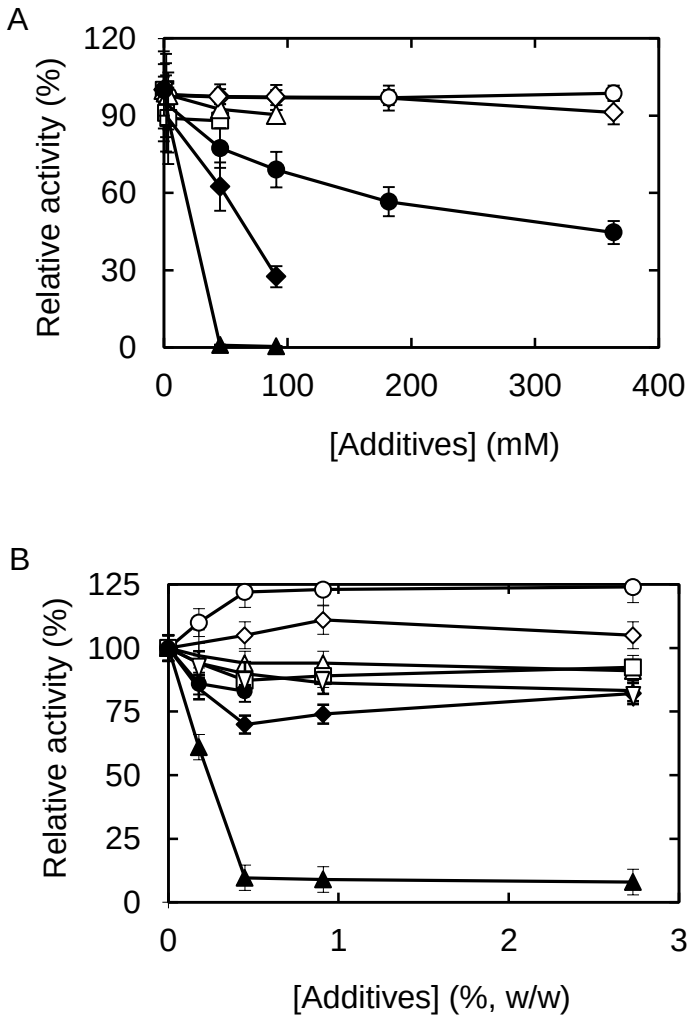

Fig. 8. Effects of various concentrations of additives on the activity of WBA at 25°C. The concentrations were in mM and the symbols show additives (A): Gly, ○; NaCl, ◇; Asp, □; Cys, Δ; glucose, ●; GSH, ◆; and Arg, ▲. The concentrations are in % (w/w) and the symbols show additives (B): ethanol, ○; DMF, ◇; DMSO, Δ; EG, □; gelatin, ●; glycerol, ◆; βME, ▲; and 2M2B, ∇. The relative activity (%) of the enzyme obtained without additive was set as 100%.

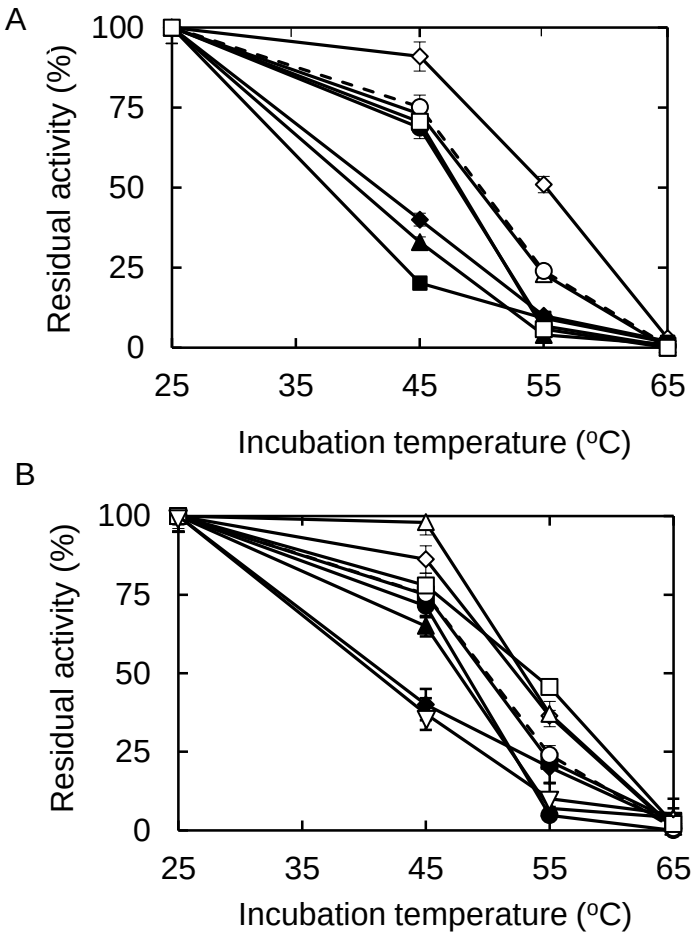

Fig. 9. Effects various additives on the thermal stabilization of WBA. Symbols for concentrations (mM) of additives (A): buffer, ○; 182 glycine, ◇; 45.5 Arg, □; 45.5 Asp, Δ; 182 NaCl, ●; 182 glucose, ◆; 45.5 Cys, ▲; and 91 GSH, ■. Symbols for concentrations (%, w/w) of additives (B): buffer, ○; 5.5 EG, ◇; 0.18 gelatin, Δ; 5.5 glycerol, □; 0.91 □; ME, ●; 2.7 DMF, ◆; 0.91 ethanol, ▲; 5.5 DMSO, ■; and 5.5 2M2B, ∇. Low concentrations of some additives were evaluated because of their solubility limit in buffer A (pH 5.4) at 25°C. The residual activity of WBA without additive was depicted in broken lines. The stability study was after 30 min of incubation.

Chapter 2

Kinetic and Thermodynamic Analysis of the Inhibitory Effects of Maltose, Glucose, and Related Carbohydrates on Wheat β-Amylase

Introduction

BA is a member of family 14 of the sequence-based classification of glycoside hydrolases (*1*). The subsite affinities of BAs were evaluated in WBA by Kato *et al*. (*31*) and in SBA by Suganuma *et al*. (*52*). According to their reports, subsite 1 has the highest affinity to a glucose unit of the substrate among the five evaluated subsites and it plays an important role in the activity of the enzyme.

The inhibition kinetics of BAs by glucose (*53*), maltose and cyclohexa-amylose (*54, 55*) were examined. However, the binding sites of these inhibitors have not been established at the subsite level. The inhibition type by glucose, cyclohexa-amylose, and maltose on SBA was pH dependent (*13*). BBA, SBA, and WBA are well characterized plant BAs. The degrees of thermo-stability defined by T_{50}, are 57^{o}C for BBA, 63^{o}C for SBA (*26*) and 50^{o}C for WBA in Chapter 1. We reported the effects of additives (carbohydrates, amino acids, organic solvents, proteins, detergents, salts, sugars, etc.) on the activity and stability of WBA in Chapter 1. It is interesting to note that 182 mM glycine and 0.18% gelatin stabilize WBA by increasing its T_{50} by 5^{o}C, and that ethanol enhance the activity of WBA by 24%, and low concentration of DMF slightly enhanced it. However, most of the evaluated additives in solvent modification in previous study

have no effects on stability, and have a decreasing effect on the activity. Among the additives examined, arginine, βME, glucose, and maltose inhibited WBA strongly as reported in Chapter 1. These lines of evidences suggest that the stability and activity of WBA could be changed by modification of the enzyme reaction system or solvent engineering.

The interaction of WBA with glucose and maltose is described by inhibition kinetics in this study. The temperature- and pH-dependences of the K_i and thermodynamic parameters for the dissociation constants of the WBA-glucose and WBA-maltose complexes are shown. In addition to the inhibitory effects of glucose and maltose as the direct end-products of starch hydrolysis, the inhibition of WBA by fructose, difructose, sucrose, trehalose, cellobiose, acarbose, and 1-deoxynojirimycin were also examined. This study provides valuable information on the end-product inhibition of WBA in the process of starch hydrolysis and also on the interaction of WBA with various related carbohydrates.

Materials and Methods

Materials - Himaltosin GS (Lot 2S24A), a commercial preparation of WBA, was purchased from HBI Enzymes (Osaka, Japan). WBA was purified from the Himaltosin preparation according to the method described previously in Chapter 1 to a homogeneous state as judged by SDS-PAGE with a molecular mass of 57.7 kDa. The preparation is composed of only WBA as a protein component. There was no starch carried over from the stabilizer starch contained in the Himaltosin preparation into the

purified WBA preparation as examined by the starch-iodine reaction. Therefore, the preparation was treated as WBA without further purification as mentioned in Chapter 1.

The molecular mass of 57.7 kDa for WBA was used to determine the molar concentration of WBA (*31*). The substrate has a weight-average molecular weight of 1.0 x 10^6 according to the manufacturer, and thus the average degree of polymerization of the glucose unit is estimated to be 6,000. Soluble starch (Lot M7H1482), maltose (Lot M1B6462), glucose (Lot M3G8543), potassium iodide (Lot V1P5303), cellobiose (Lot M2G9713), and other chemicals were purchased from Nacalai Tesque (Kyoto, Japan) and iodine solution (Lot CEM7810), acarbose (Lot LAQ5872), difructose anhydride (Lot TLM1444), α,α-1,1-trehalose (Lot PEH6208; hereinafter designated as simply trehalose), and 1-deoxynojirimycin (Lot DCL2444) were from Wako Pure Chemical (Osaka, Japan).

Inhibition of WBA by glucose, maltose, and other carbohydrates - WBA (1.6 μM) and various concentrations of glucose, maltose, and other carbohydrates were prepared in buffer A. The WBA solution (100 μl) was pre-incubated with 100 μl of the carbohydrates solutions at the initial concentrations of 0, 0.15, 0.31, 0.62, 1.23, and 2.33 M glucose; 0.15, 0.31, and 0.62 M maltose; 0.15, 0.31, 0.46, 1.17, and 1.75 M fructose; 0.04, 0.08, 0.12, 0.15, and 0.24 M difructose; 0.15, 0.31, 0.46, and 0.62 M sucrose; 0.15, 0.31, 0.46, 0.62, 1.11, and 1.66 M trehalose; 0.14, 0.28, and 0.42 M cellobiose; 0.004, 0.040, 0.080, 0.120, and 0.150 M acarbose; and 0.001, 0.002, 0.005, and 0.009 M 1-deoxynojirimycin for 5 min at 25°C before reacting with the substrate. The soluble starch solution 0.69% (w/v; 450 μl) was mixed with the mixture (200 μl) of WBA and the carbohydrates and was hydrolyzed for 5 min in buffer A at 25°C. Therefore, the

WBA and soluble starch concentrations in the initial conditions of the enzyme reaction were 0.25 μM and 0.69 % (w/v), respectively. The reaction was stopped by mixing the reactants (50 μl) with 4.5 ml of 1 mM potassium iodide (KI) prepared in 0.1 N HCl. The absorbance was measured at 580 nm using a Beckman-Coulter DU 800 spectrophotometer (Batavia, IL, USA) (*13, 56*). Iodine staining is not preferable method for precise kinetic studies. Nevertheless, it was not possible to use the common product measurement methods such as neocuproine, DNS (dinitrosalicylic acid), and Somogi-Nelson (*57*) methods which are based on the determination of the reducing ends of the products because some of the carbohydrates examined possess reducing ends. The 50% inhibitory concentrations (IC_{50}) of the carbohydrates are their concentrations giving 50% of the activity observed in the absence of the carbohydrates.

Reversibility of WBA inhibition by glucose and maltose - Reversibility of the WBA inhibition by glucose and maltose was examined through dialysis and dilution techniques. In dialysis, 1.6 μM of WBA in buffer A was filtered through Millipore membrane filter (Type HA; pore size: 0.45 μm) and kept in ice for immediate use. The filtered WBA solution (1 ml) was incubated for 5 min with 1 ml of buffer A, or 1 ml of 2 M glucose or 0.5 M maltose prepared in buffer A. The WBA-glucose or WBA-maltose mixtures were dialyzed using a dialysis membrane (Wako Chemicals, Kyoto) against buffer A 100-times the volume of the mixtures; the dialyzing buffer was changed 3 times with 2 h intervals at 4^{o}C. Soluble starch (0.82%, w/v) was hydrolyzed by WBA for 10 min at 25^{o}C. The reaction rate of substrate hydrolysis was determined using iodine staining method and the reaction rates of the dialyzed WBA in the presence of glucose and maltose were compared with the reaction rates in the absence of the

inhibitors.

In the dilution method, the concentration of WBA 10 times higher than that described above was incubated with buffer A or with buffer A containing glucose or maltose at the initial concentrations of 18 mM and 0.18 M. After pre-incubation for 5 min, the WBA mixture with 0.18 M of glucose or maltose was 10 times diluted to 18 mM glucose or maltose with buffer A. The concentrations of the inhibitors (glucose and maltose) in the buffer were adjusted to attain the mentioned concentrations after dilution and 0.14 μM WBA. The relative activities in the presence of the carbohydrates were calculated relative to the activity in the absence of the carbohydrates and the reversibility (%) of the 10 times diluted forms was calculated relative to the same lower concentrations of the carbohydrates before dilution (*41*). The same method was repeated to check the reversibility of WBA inhibition in the presence of various concentrations of glucose or maltose at pH 5.4, 25^{o}C.

Temperature-Dependence of the K_i for the inhibition of WBA by glucose and maltose - Various initial concentrations of carbohydrates in the enzyme reaction: 0.08, 0.15, 0.23, 0.31, 0.38, and 0.46 M glucose; 0.07, 0.08, 0.12, 0.15, 0.18, and 0.23 M maltose were prepared in buffer A at 15, 25, 35, and 45^{o}C. Various initial concentrations of soluble starch: 0.35, 0.52, and 0.69% (w/v) were hydrolyzed by 0.32 μM WBA pre-incubated for 5 min with various concentrations of glucose or maltose at each temperature. The initial velocity (*v*) of starch hydrolysis were determined from the reaction progress over 0.5-4.0 min and the reactant (50 μl) was stopped by adding 4.5 ml of the 1 mM iodine solution. The K_i values at each temperature were determined from the Dixon plots (*58*). The enthalpy changes (ΔH^{o}) of the dissociations of the

WBA-glucose and WBA-maltose complexes were determined from the van't Hoff plots (*59*), while the Gibbs energy changes (ΔG^o), and entropy changes (ΔS^o) were determined from Eqs. 1 and 2 (*59, 60*).

$$\Delta G^o = -RT \ln K_i \qquad (1)$$

$$\Delta S^o = (\Delta H^o - \Delta G^o)/T \qquad (2)$$

where R is the gas constant and T is temperature in Kelvin

pH-Dependence of K_i - Various initial concentrations of soluble starch: 0.35, 0.69, 1.04, 1.38, 1.73, and 2.08% (w/v) were prepared in 20 mM glycine-HCl buffer (pH 3.0), buffer A, and 20 mM borate buffer (pH 9.0) at 25^{o}C following the method previously reported (*61*). WBA (0.32 μM) in the respective buffers at each pH was pre-incubated with 0.31, and 0.62 M glucose; and 0.15, and 0.31 M maltose for 4 min at 25^{o}C. The soluble starch solution (450 μl) varying in concentration was mixed with 100 μl of the pre-incubated WBA-glucose or WBA-maltose mixtures at pH 3.0, 5.4, and 9.0 at 25^{o}C. The initial velocity (v) were determined from the reaction progress over 5 min. The absorbance was measured at 700 nm using a Beckman-Coulter DU 800 spectrophotometer. The inhibition type and K_i values at each pH was determined from the Hanes-Woolf plots (*62*). The K_i values were calculated using Eqs. 3 and 4 for competitive and uncompetitive inhibitors, respectively (*63*).

$$K_i = [I]_o / ((K_{m,app}/K_m) - 1) \qquad (3)$$

$$K_i = [I]_o / ((V_{max}/V_{max,app}) - 1) \qquad (4)$$

where, K_m and V_{max} are the Michaelis constant and maximum reaction rate observed in the absence of inhibitors; $K_{m,app}$ and $V_{max,app}$ are those in the presence of inhibitors; and

$[I]_o$ is inhibitor initial concentration.

Results

Inhibition of WBA by glucose, maltose, and other carbohydrates - The rate of soluble starch hydrolysis by WBA was measured from the decrease in absorbance at 580 nm as described in the Materials and Methods. The catalytic activity of WBA was reduced by increasing the concentrations of maltose or other carbohydrates (Figs. 1). The inhibitory activities of fructose, sucrose, trehalose, difructose (α-D-fructofuranose-β-D-fructofuranose-2',1:2,3'-dianhydride shown in Fig. 2), cellobiose, and 1-deoxynojirimycin were comparatively weak. On the other hand, acarbose and maltose strongly inhibited WBA activity followed by glucose (Fig. 3A). The inhibition by acarbose, maltose, and glucose were significant with the IC_{50} values of 0.06 ± 0.01, 0.22 ± 0.09, and 1.41 ± 0.17 M, respectively. The IC_{50} values were not attained by the other carbohydrates examined at the concentrations of their maximal solubility (Fig. 3B). The maximum degrees of inhibitions by 1-deoxynojirimycin, difructose, trehalose, sucrose, fructose, and cellobiose were around 30%, and the IC_{50} values were estimated to be: 9 mM, 0.3 M, 2 M, 0.5 M, 2 M, and 0.4 M, respectively. The IC_{50} values were the values of $[I]_o$ at which the fractional activity of WBA becomes 0.5 as shown in Fig. 3.

Reversibility of WBA inhibition by maltose and glucose - The reversibility of WBA inhibition by glucose and maltose was studied through both dialysis and dilution

methods. The activity of WBA in the absence and presence of inhibitors was measured using blue value method (ΔA_{580}) after dialysis. The activity of WBA in buffer A (control) was taken as 100%, and the reversibility in the presence of inhibitors after dialysis were calculated relative to the activity of the control (Table 1). The activity of WBA was completely restored through dialysis.

In the dilution method, the inhibitor concentrations were diluted 10 times and the effect of dilution was examined following the method described previously (*41*). The activity of WBA in the presence of 18 mM maltose was 86% relative to the activity observed in the absence of inhibitors. When 180 mM maltose was diluted to 18 mM, the activity recovered to 89%, and hence the reversibility was 103 ± 6%. In the same manner, various concentrations of glucose and maltose were studied and complete reversibility was observed through dilution (Table 2).

Temperature-Dependence of K_i - The temperature-dependence of the K_i values of glucose and maltose at 15, 25, 35, and 45^{o}C were estimated at pH 5.4 using Dixon plots (Figs. 4 and 5). The K_i values of both inhibitors increased slightly with increasing temperature, indicating that the inhibitory activity of the inhibitors decrease with increasing temperature in the range of 15-45^{o}C. The thermodynamic parameters: the Gibbs energy change (ΔG^o), enthalpy change (ΔH^o), and entropy change (ΔS^o) of the dissociation of the WBA-glucose or WBA-maltose complexes were determined at various temperatures (Table 3). The van't Hoff plots of the K_i values of WBA inhibition by glucose and maltose with the slope $-\Delta H^o/R$ were shown in Fig. 6.

pH-Dependence of K_i - The inhibition types and K_i values of WBA inhibition by

glucose and maltose at 25°C were evaluated at pH 3.0, 5.4, and 9.0 using the Hanes-Woolf plots shown in Figs. 7 and 8. Here, we should note the difference between the inhibitory mechanisms of the competitive inhibition and uncompetitive inhibition. In the case of competitive inhibition, the inhibitor (I) binds only to the enzyme (E) to form the enzyme-inhibitor complex (EI) but does not bind to the enzyme-substrate complex (ES). In the case of uncompetitive inhibition, it binds only to the ES complex to form the enzyme-substrate-inhibitor (ESI) ternary complex but does not bind to E. Thus, the EI complex is not formed in uncompetitive inhibition, whereas the ESI complex is not formed in competitive inhibition. In other words, it binds to the active site of the enzyme in competitive inhibition, but binds to the secondary binding site other than the active site in uncompetitive inhibition. Glucose and maltose bind to the active site of WBA at pH 3.0 and 5.4, and bind to the secondary binding site of WBA at pH 9.0. In competitive inhibition, K_m increases and V_{max} remains constant while both the K_m and V_{max} values decrease in uncompetitive inhibition. It is interesting to note that the K_i values obtained at pH 5.4 for both glucose and maltose are slightly larger than at pH 3.0 and 9.0, suggesting that the inhibitory effects of glucose and maltose on WBA at pH 5.4 (which is the optimal pH of WBA) are lower than at pH 3.0 and 9.0, at which the enzyme activity is extremely low. The K_i values determined by the Hanes-Woolf plot and Dixon plot for the respective inhibitors at pH 5.4 and 25°C are in good agreement. The K_i values of glucose and maltose in the WBA inhibition are considered to be the dissociation constant (K_d) values for the WBA-glucose and WBA-maltose complexes. Thus, the ΔG^o values of the dissociations of the WBA-glucose and WBA-maltose complexes can be determined from K_i values at each pH (Table 4).

Discussion

Inhibition of WBA - The catalytic activity of WBA was noticeably inhibited in a dose-dependent manner by acarbose, maltose and glucose. BAs from other crops such as soybean (*13*), sweet potato (*64*), and alfalfa (*56*) were reported to be inhibited by glucose and maltose. In the present study, other sugar analogues and sugar derivatives were systematically selected to evaluate their inhibitory effects on WBA, among which, only acarbose containing acarviosin (α-amylase inhibitor) and maltose moiety was found to expressively inhibit WBA. However, this IC_{50} value of acarbose is very big in view of pharmaceutical industries. The well-known α-amylase inhibitor, acarbose was reported to competitively inhibit BBA (*65*). Acarbose analogues, containing cellobiose and lactose structures were potent competitive inhibitors of β-glucosidase, and the lactose analogues were uncompetitive inhibitors of β-galactosidase (*66*). On the other hand, fructose, sucrose, trehalose, difructose, 1-deoxynojirimycin, and cellobiose did not show substantial inhibition on WBA. However, the maximum solubility of cellobiose is very low even at high temperature and hence, difficult to evaluate its inhibitive power.

The respective IC_{50} and K_i values of maltose are 0.22 ± 0.09 and 0.12 ± 0.03 M and that of glucose are 1.41 ± 0.17 and 0.33 ± 0.02 M at 25^{o}C, pH 5.4 (Fig. 3 and Table 3). The K_i values of 0.034 ± 0.0 M for maltose and 0.32 ± 0.08 M for glucose were reported in SBA inhibition (*13*). This shows that maltose is stronger inhibitor than glucose of not only WBA but also of SBA. From the crystal structure study of maltose and glucose binding of BacBA, it was stated that glucose binds to subsite 1 and maltose binds to

subsites 1 and 2 of the active site (*2*). This attests that maltose binds at two subsites of BacBA at a time while glucose binds at only a single subsite. Therefore, the molar energy of maltose binding to the active site may be given as the sum of the molar energy of glucose moieties binding to subsites 1 and 2, while the molar energy of glucose binding is that to subsite 1. Hence, the inhibition of maltose is stronger than that of glucose. This is not always true if more than one maltose molecules that bind different binding sites or subsites are considered. In such cases, the binding affinities of the second binding site or subsites are enhanced in positive co-operativity and inhibited in negative co-operativity. By considering the binding modes of glucose and maltose by WBA to be similar to that of BacBA, we can estimate the molar binding energy of the glucose moieties to subsites 1 and 2 separately from Table 4. The binding energy of the glucose moiety to subsite 1 would be -3.43 and -2.33 kJ mol^{-1} at pH 3.0 and 5.4, respectively, and those to subsite 2 would be -1.81 and -2.20 kJ mol^{-1}. From these values, it can be seen that the molar binding energy to subsite 1 is much lower in magnitude at pH 5.4 than at pH 3.0, although that to subsite 2 is higher at pH 5.4 than at pH 3.0, and the molar binding energy of maltose to the active site of WBA is slightly smaller at pH 5.4 than at pH 3.0 as shown in Table 4.

Reversibility of maltose and glucose inhibition - Inhibition of WBA by glucose and maltose was completely reversible. We can also infer from reports (*13, 55, 56*) that the inhibition was reversible indicating that the molecular interaction between WBA and the sugars is not covalent. The reversibility of WBA inhibition by glucose and maltose was examined by dialysis. The dialysis was conducted at $4^{o}C$ to avoid activity loss, and WBA activity was completely reinstated (Table 1). In dialysis, enzymes are restored

from the easily dissociable, non-covalently formed EI (*67*). The reversibility of WBA activity was also confirmed through dilution technique. Similar dilution principles were reported on the reversibility study of the inhibition of thermolysin (*41*) and neuraminidase (*68*) by alcohols. Contrary to the dialysis method, low concentration of the inhibitor remains in the reaction after dilution. Due to this fact, reversibility was calculated relative to the initially low inhibitor concentration the same as after 10 times dilution. It was explained that the drastic activation of neuraminidase activity after dilution could be because of the change in the conformation and hydrated state of the enzyme, in addition to the change in the viscosity of the reaction medium (*68*).

Temperature-Dependence of K_i - The K_i values of EI dissociations were affected by the change in reaction temperature which influences the molecular activities of the solvent, enzyme, and inhibitors. It also affects the structure of the protein, which entails change in EI binding or dissociation (*60, 69*). The K_i values increased with temperature (Table 3) which agrees with reports on various enzymes and inhibitors showing an increase in K_i with temperature (*41, 59, 60, 69*). The K_i values at various temperatures were determined from the Dixon plots in this study following the method previously reported (*58, 70*). Kitagishi *et al.* (*69*) have recommended that inhibitor concentrations close to the K_i values should be used in order to determine the K_i values accurately and in conditions where $[E]_o << [I]_o$, the K_i values can be obtained from the Dixon plot. The concentration of enzyme is much less than the inhibitor concentrations in our study.

Temperature variation exerted significant influence on the thermodynamic parameters of the EI complexes dissociations (Table 3). The positive ΔH^o values of the dissociations of the WBA-glucose and WBA-maltose complexes indicate that the

dissociations were endothermic. The ΔG^o, a state function reaction change of the system determines the relative importance of the enthalpy and entropy terms as driving forces behind a particular reaction (*71*). The positive values of ΔG^o indicate non-spontaneous dissociations of the EI complexes. Since the enthalpy terms are greater in magnitude than the entropy terms, the EI dissociations are enthalpy-driven. The ΔG^o values were found to decrease with increasing temperature. The source of the energy demand for the dissociations is the partial hydrogen-bond breakage in the system (*72*). The bindings of glucose and maltose to the active site of BacBA are mainly by hydrogen-bonds from the crystallographic study (*2*). It can be elucidated from these facts that there is hydrogen-bond cleavage with the dissociations of the WBA-glucose or WBA-maltose complexes. In general, the hydrogen-bond dissociation energies are 20 kJ mol^{-1} but it can span more than two orders of magnitude (about 8-100 kJ mol^{-1}) depending on the nature of the interactions and environments (*72*). The ΔG^o required for the dissociation of the WBA-maltose complex is almost twice as that of the WBA-glucose complex (Tables 3 and 4), which is in good agreement with the structural compositions of the inhibitors.

pH-Dependence of K_i - The inhibition type of WBA by both glucose and maltose was affected by pH. Both sugars behaved as competitive inhibitors at pH 3.0 and 5.4 at 25^{o}C but exhibited uncompetitive type of inhibition at pH 9.0. Nomura *et al*. (*13*) reported that the inhibition type on SBA by maltose and cyclohexa-amylose was competitive at pH 5.4 but it was mixed-type by glucose at pH 5.4 (I binds both E and ES to form EI and ESI complexes), and competitive by both the sugars at pH 8 and form solely EI complexes. Change of the inhibition type based on pH was also reported

in aspartic protease (*73*) and pepsin (*74*). The isoelectric point (p*I*) value of WBA was reported to be 5.8 (*75*). The pK_{e1} (where the ionizable group 1 of SBA is deprotonated) and pK_{e2} (where the ionizable group 2 of SBA is protonated) were 3.41 and 8.09 with p*I* value of 5.63 (*32, 76*). This is to indicate that at pH 3.0, WBA has a net positive charge. In this state, both glucose and maltose bind to the active site. At pH 9 when the net charge of WBA becomes negative, the sugar molecules bind the secondary binding sites on the EI complex and behaved as uncompetitive inhibitors. According to the subsite model of amylases (*31, 77*), the inhibition type depends on the binding site of the inhibitors (*78*).

The K_i values of the dissociations of the WBA-glucose and WBA-maltose complexes were also pH dependent (Table 4). The pH-dependences of K_i in various enzymes and inhibitors were reported with the changes in K_i being different depending on the response of particular enzymes to the pH changes (*13, 58, 69, 73, 74*). This phenomenon apparently suggests that pH either induces conformational or change in ionization states of binding residues of an enzyme. Our result in Table 4 shows that the inhibitive power of the inhibitors declines with increasing the activity of the enzyme at optimum pH.

In starch-saccharifying industries, maltose and glucose are continuously produced from the enzymatic starch hydrolysis process. Hence, studying the molecular interactions of these end-products and BA is worthwhile to search for suitable ways of enhancing production. This study thus, provides valuable information on the end-product inhibition of WBA, which has direct implication on the efficiency and cost of production. Mechanisms of reducing the inhibitory actions of glucose and maltose can be suggested like, continuous removal of the end-products from the reaction.

In conclusions, the end products of starch hydrolysis, maltose and glucose inhibit WBA dose-dependently and the interaction is reversible. The catalytic activity of WBA is more strongly inhibited by acarbose than by maltose and glucose. The K_i of glucose and maltose are temperature dependent. The dissociations of the WBA-glucose and WBA-maltose complexes are endothermic. Both glucose and maltose bind the active site when WBA is protonated at pH 3.0 and 5.4 and to the secondary binding sites when its net charge becomes negative at pH 9.0. A change in pH affects the active site integrity in binding the inhibitors or induces a conformational change to WBA and hence, affects the type of inhibition. Therefore, both the temperature and pH of starch hydrolysis operations require careful manipulation to minimize end-product inhibition in starch-saccahrifying industries.

Table 1. The initial velocity and relative activity of WBA in the hydrolysis of soluble starch in the presence of glucose and maltose before and after dialysis.

	Control (buffer A)	0.36 M glucose	0.09 M maltose
v before dialysis	0.034	0.020	0.017
(Relative activity)	(100%)	(59%)	(50%)
v after dialysis	0.030	0.030	0.029
(Relative activity)	(100%)	(100%)	(97%)

The initial concentrations of WBA and soluble starch in the reaction solution were 0.14 μM and 0.82% (w/v), respectively. The activity of WBA pre-incubated with buffer A was considered as 100% and the activities in the presence of inhibitors were calculated relative to the control (buffer A).

Table 2. The relative activity and reversibility of WBA pre-incubated with various concentrations of glucose and maltose in hydrolysing soluble starch before and after 10 times dilution.

	$[I]_o$			Reversibility (%)
	0.02 M	0.18 M	0.18 M →0.02 M	
Glucose	82 ± 11	74 ± 6	85 ± 3	104 ± 3
Maltose	86 ± 3	26 ± 12	89 ± 2	103 ± 6
	$[I]_o$			
	0.03 M	0.27 M	0.27 M →0.03 M	
Glucose	97 ± 7	68 ± 6	99 ± 4	102 ± 12
Maltose	86 ± 8	15 ± 1	90 ± 17	105 ± 18

The initial concentrations of WBA and soluble starch in the reaction solution were 1.6 μM and 0.82% (w/v), respectively. The activity of WBA in the absence of inhibitors was taken as 100%. Relative activity is the activity relative to the activity in the absence of inhibitors while reversibility is relative to the activity of the same lower inhibitor concentrations before dilution as the diluted forms. The values are mean ± SD. Each experiment was repeated three times.

Table 3. The temperature-dependence of K_i and thermodynamic parameters of the EI dissociations in WBA inhibition by glucose and maltose at pH 5.4.

		Temperature (K)			
		288	298	308	318
Glucose	K_i (M)	0.26 ± 0.05	0.33 ± 0.02	0.38 ± 0.03	0.42± 0.04
	ΔG^o (kJ mol^{-1})	3.22 ± 0.97	2.74 ± 0.51	2.47± 0.69	2.29 ± 0.93
	ΔS^o (J mol^{-1} K^{-1})	30.73 ± 2.95	31.31 ± 2.85	31.17 ± 2.76	30.75 ± 2.67
	$T\Delta S^o$ (kJ mol^{-1})	8.85 ± 0.85	9.33 ± 1.06	9.60 ± 0.74	9.78 ± 0.96
Maltose	K_i (M)	0.11 ± 0.05	0.12 ± 0.03	0.14 ± 0.04	0.18 ± 0.04
	ΔG^o (kJ mol^{-1})	5.28 ± 0.91	5.24 ± 0.77	5.03 ± 1.09	4.53 ± 0.92
	ΔS^o (J mol^{-1} K^{-1})	24.41 ± 3.58	23.72 ± 3.29	23.64 ± 3.57	24.47 ± 2.96
	$T\Delta S^o$ (kJ mol^{-1})	7.03 ± 1.03	7.07 ± 0.98	7.28 ± 1.10	7.78 ± 0.94

ΔH^o was determined from van't Hoff plot [ln K_i *vs.* 1/T (K^{-1})]; where the slope of the graph equation gives -$\Delta H^o/R$ and 12.07 kJ mol^{-1} for inhibition by glucose, and 12.31 kJ mol^{-1} for inhibition by maltose. Whereas ΔG^o and ΔS^o were calculated as described in the Materials and Methods.

Table 4. The pH-dependence of K_i, ΔG^o, and inhibition types for the dissociations of EI in the inhibition of WBA by glucose and maltose at 25°C.

		pH		
		3.0	5.4	9.0
Glucose	K_i (M)	0.25 ± 0.03	0.39 ± 0.03	0.21 ± 0.03
	ΔG^o (kJ mol^{-1})	3.43 ± 0.13	2.33 ± 0.15	3.86 ± 0.34
	Inhibition type	competitive	competitive	uncompetitive
Maltose	K_i (M)	0.12 ± 0.04	0.16 ± 0.03	0.11 ± 0.04
	ΔG^o (kJ mol^{-1})	5.24 ± 0.13	4.53 ± 0.07	5.46 ± 0.10
	Inhibition type	competitive	competitive	uncompetitive

The values are mean ± SD, each experiment was done in triplicates. The types of inhibition by glucose and maltose are from the Hanes-Woolf plots at various temperatures shown in Figs. 7 and 8.

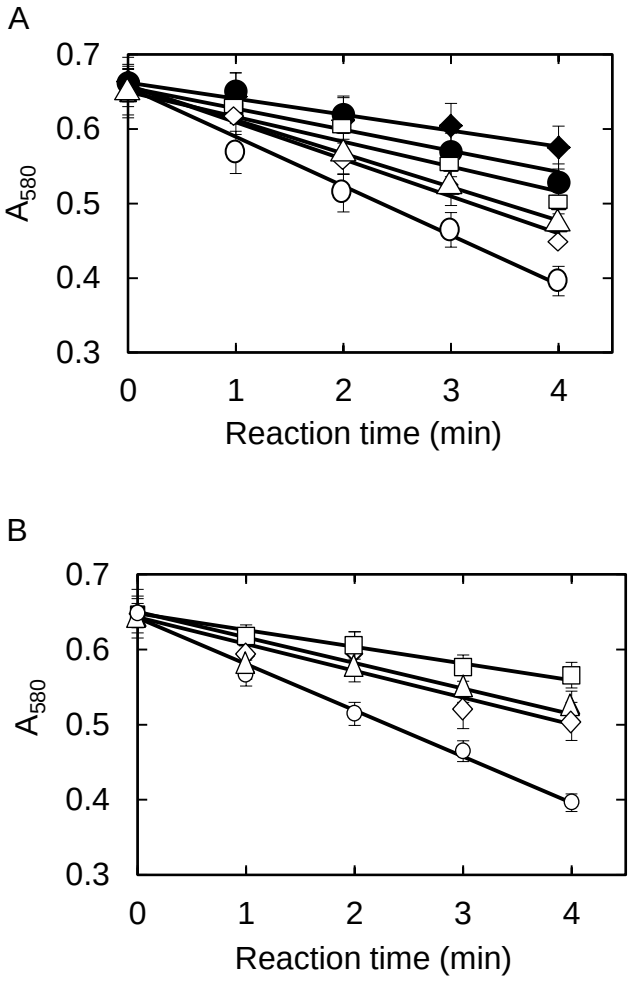

Fig. 1. Inhibition of WBA activity by various concentrations of glucose and maltose. The initial concentrations of WBA and soluble starch (substrate) in the reaction solution were 0.25 μM and 0.82%, (w/v), respectively. The initial concentrations of glucose (A) were: ○, 0; ◇, 0.15; Δ, 0.31; □, 0.62; ●, 1.23; and ◆, 2.33 M. The initial concentrations of maltose (B) were: ○, 0; ◇, 0.15; Δ, 0.31; and □, 0.62 M. WBA and carbohydrates (glucose or maltose) were pre-incubated for 5 min before reaction in buffer A. The progress of the reaction was followed by measuring the absorbance at 580 nm after staining the reaction solution by KI and the initial reaction rate was evaluated from the slope of the progress curve. The activity of WBA was considerably inhibited by both sugar inhibitors in dose-dependent manner.

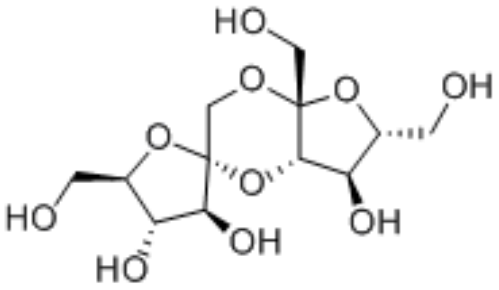

Fig. 2. The structure of difructose anhydride III (DFA). Difructose anhydride (DFA) or α-D-fructofuranose-β-D-fructofuranose-2',1:2,3'-dianhydride is the smallest cyclic disaccharide consisting of two fructose residues.

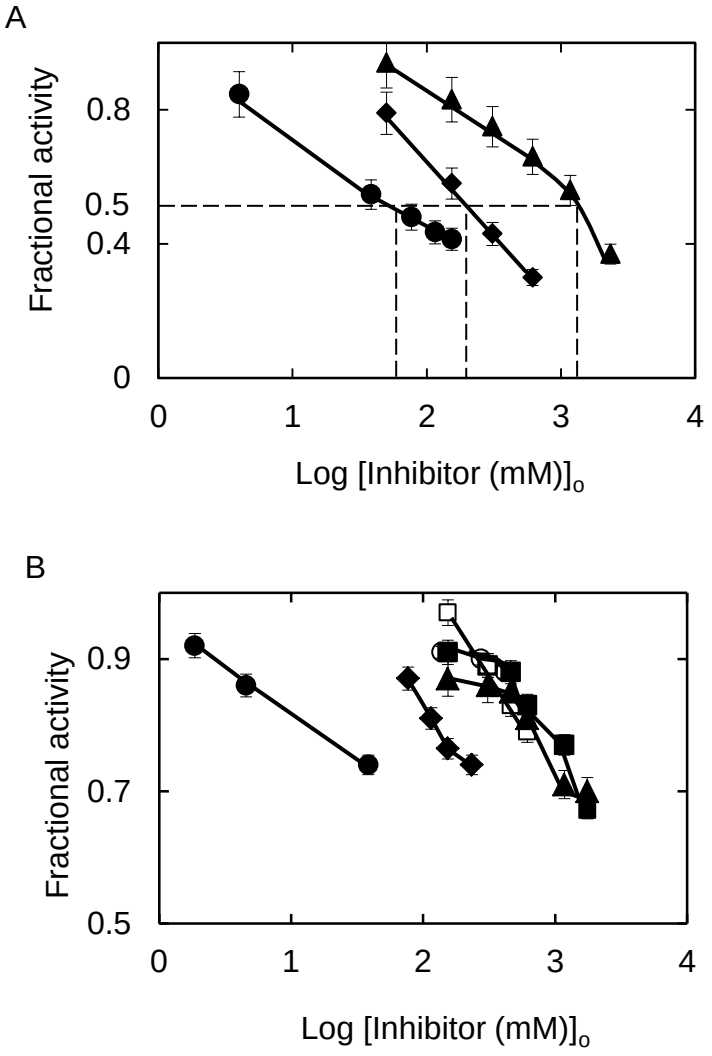

Fig. 3. **Inhibition of WBA by various carbohydrates in the hydrolysis of soluble starch.** Dependence of the relative activity of WBA on the logarithmic concentrations of various carbohydrates. The carbohydrates are (A): ●, acarbose; ◆, maltose; and ▲, glucose. The carbohydrates are (B): ●, 1-deoxynojirimycin; ■, difructose; ▲, trehalose; □, sucrose; ◆, fructose; and ○, cellobiose. The enzyme reaction was done in buffer A at pH 5.4, and 25°C. WBA activity observed in the absence of carbohydrates was set to the relative activity of 1.0. The IC_{50} of the inhibitors are the concentrations corresponding to the midpoint of the relative activities.

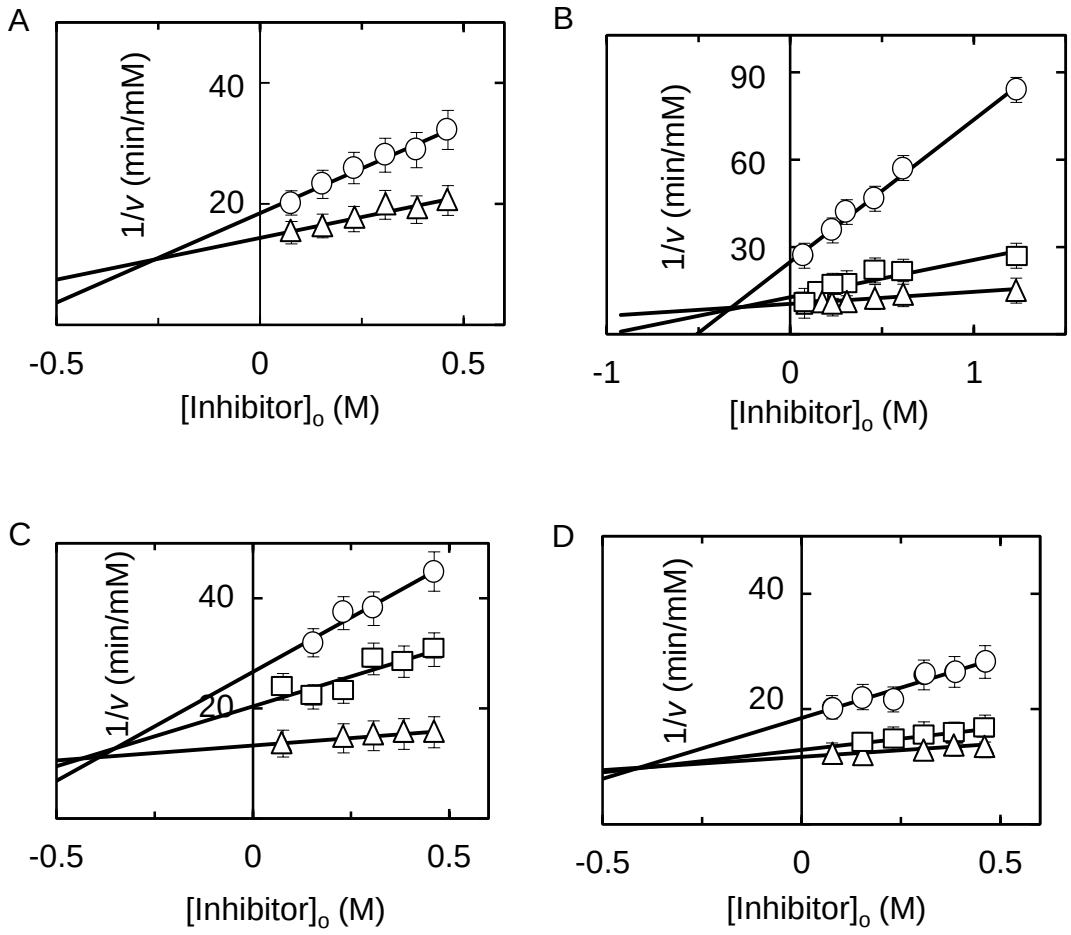

Fig. 4. Dixon plots of WBA-glucose interaction at various temperatures. Reciprocals of the initial reaction rates in the hydrolysis of soluble starch were plotted against the glucose concentrations. A, B, C, and D indicate reaction temperatures at 15, 25, 35, and 45°C, respectively at constant pH 5.4. The initial concentrations of soluble starch in the reaction: ○, 0.17; and Δ, 1.38% (w/v), at 25°C (A); and ○, 0.35; □, 0.52; and Δ, 0.69% at 15, 35, and 45°C (B, C, and D). The initial concentration of WBA was 0.32 μM. WBA was pre-incubated for 5 min with increasing concentrations of glucose and catalyzed various concentrations of soluble starch.

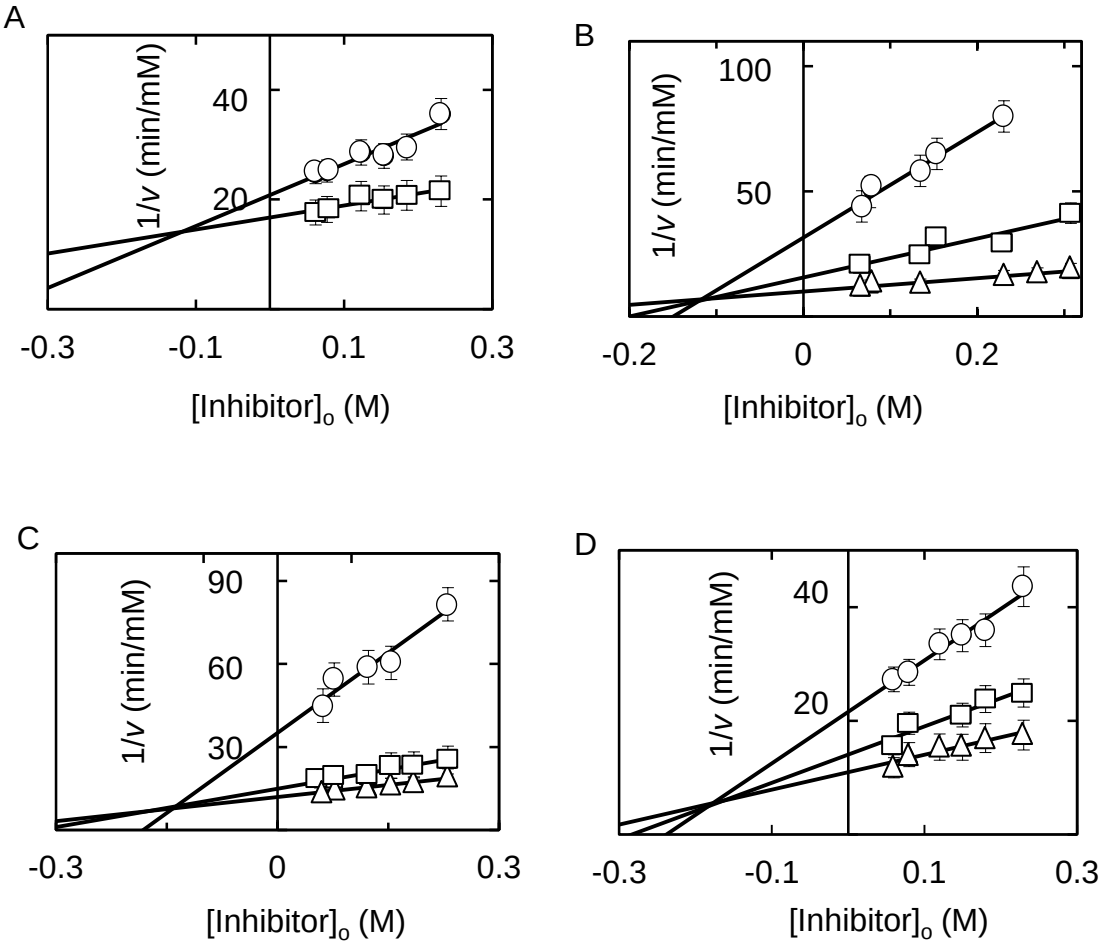

Fig. 5. Dixon plots WBA-maltose interaction at various temperatures. Reciprocals of the initial reaction rates in the hydrolysis of soluble starch were plotted against maltose concentrations. A, B, C, and D indicate reaction temperatures at 15, 25, 35, and 45^{o}C, respectively at pH 5.4. The initial concentrations of soluble starch in the reaction: ○, 0.17; and Δ, 1.38% (w/v), at 25^{o}C (A); and ○, 0.35; □, 0.52; and Δ, 0.69% at 15, 35, and 45^{o}C (B, C, and D). The initial concentration of WBA was 0.32 μM. WBA was pre-incubated for 5 min with increasing concentrations of maltose in the hydrolysis of various concentrations of soluble starch.

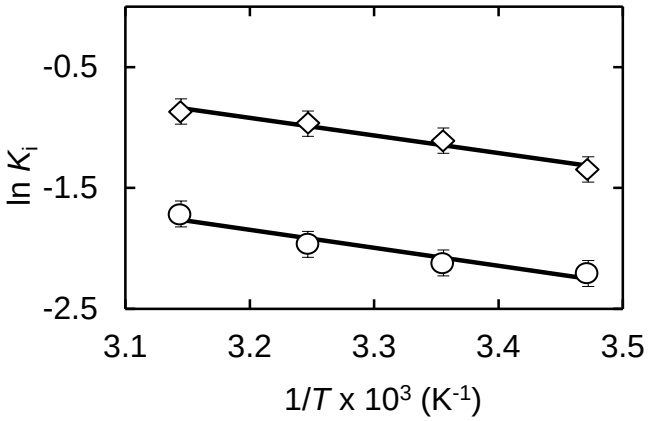

Fig. 6. van't Hoff plots WBA inhibition by glucose and maltose. The K_i of WBA-glucose and WBA-maltose dissociations were examined at various temperatures at pH 5.4. The symbols: ○, maltose; and ◇, glucose. The slope of the plot gives $-\Delta H^o/R$.

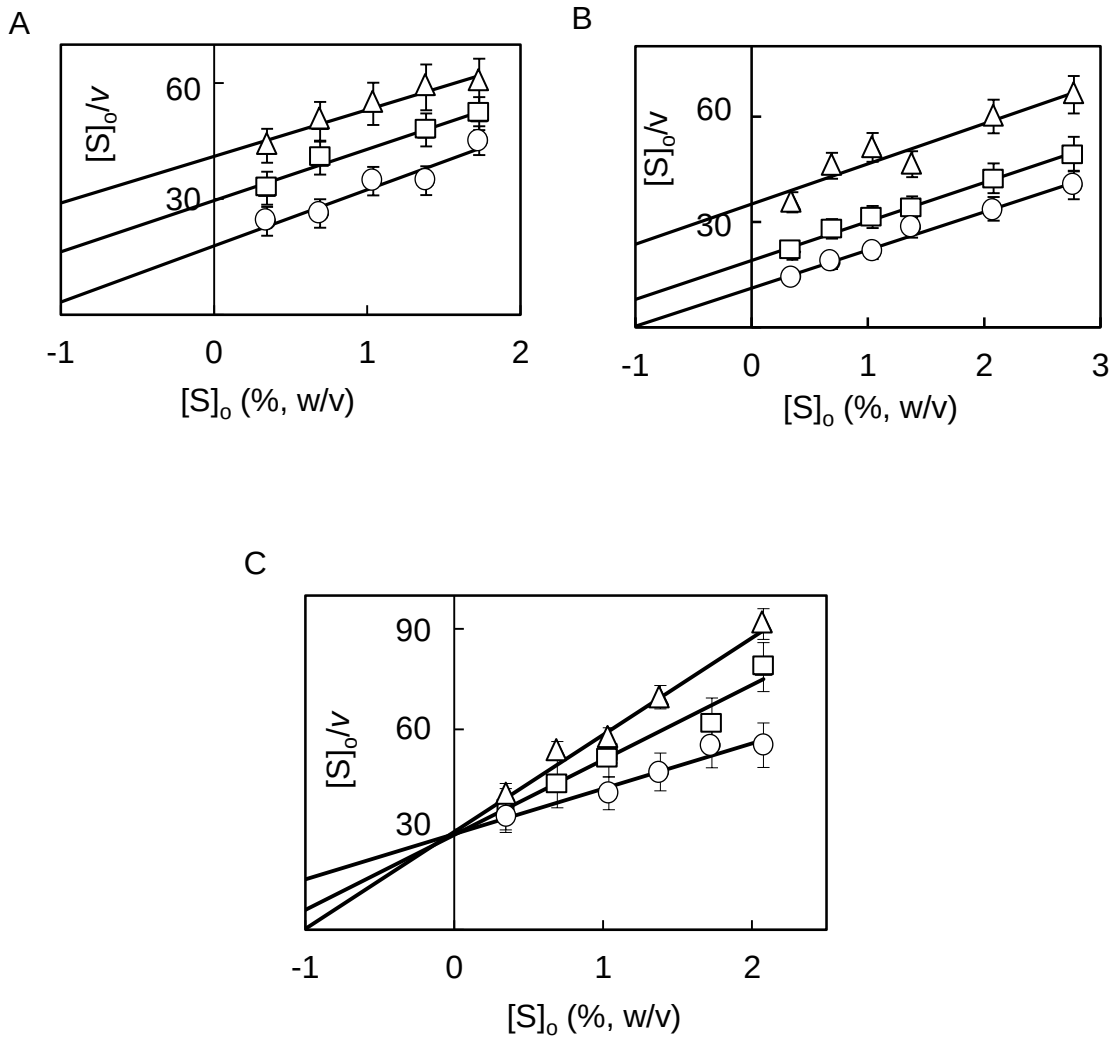

Fig. 7. Hanes-Woolf plots of initial velocity (*v*) in the presence and absence of glucose at pH 3.0, 5.4, and 9.0. The pH of the reaction: A, 3.0; B, 5.4; and C, 9.0. The initial concentrations of glucose in the enzyme reaction solution: ○, 0; □, 0.31; and Δ, 0.62 M. The inhibition type is competitive at pH 3.0 and 5.4 whereas uncompetitive type at pH 9.0, 25°C. The unit of the vertical axes is (%, w/v)/(mM min^{-1}).

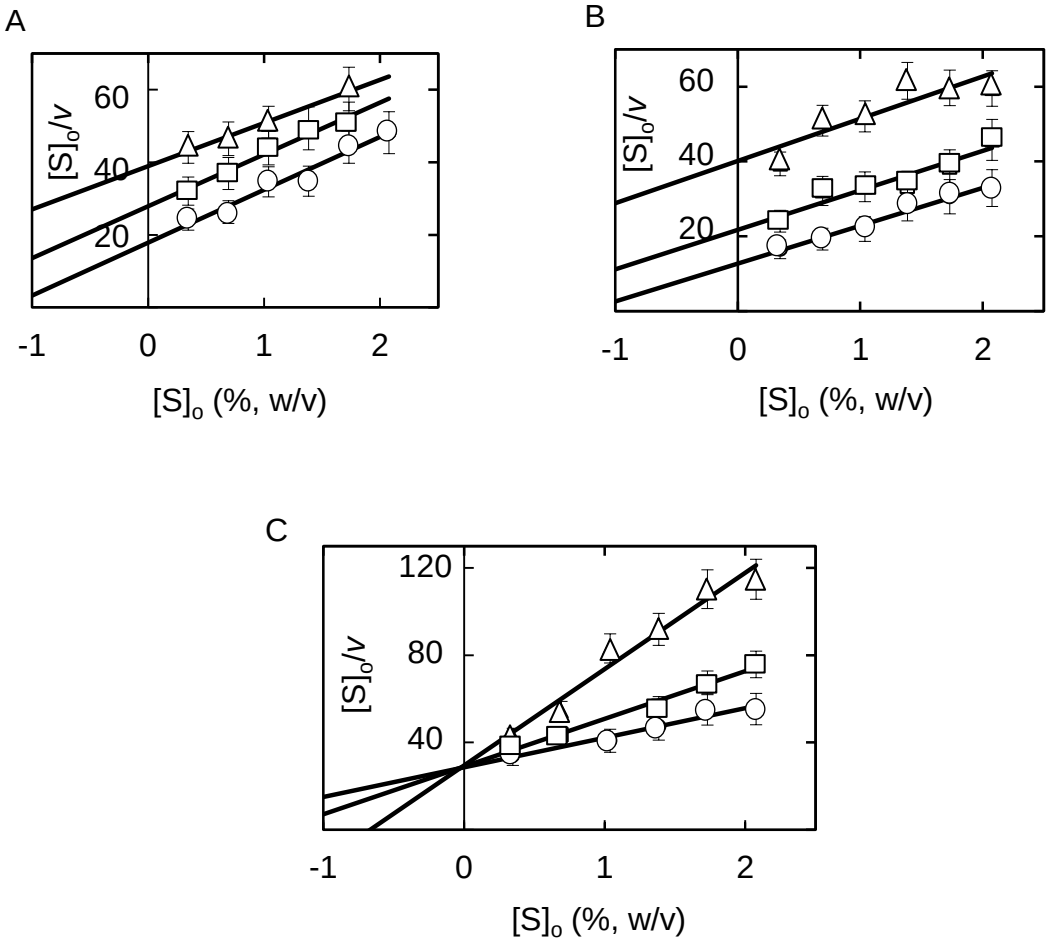

Fig. 8. Hanes-Woolf plots of the initial velocity (*v*) in the presence and absence of maltose at pH 3.0, 5.4, and 9.0. The pH of the reaction: A, 3.0; B, 5.4; and C, 9.0. The initial concentrations of maltose in the enzyme reaction solution: ○, 0; □, 0.31; and Δ, 0.62 M. The inhibition type is competitive at pH 3.0 and 5.4 whereas uncompetitive type at pH 9.0, 25^{o}C. The unit of the vertical axes is (%, w/v)/(mM min^{-1}).

Chapter 3

Interaction of Wheat β-Amylase with Maltose and Glucose as Examined by Fluorescence

Introduction

BAs are found in higher plants and some microorganisms but there are variations between bacterial and plant BAs in binding and hydrolyzing raw starch (*53*). This binding aptitude difference is credited to their starch-binding domain located at the C-terminus of their sequence (*3*). The subsite affinities of WBA (*31*) and SBA (*52*) were described. Subsite 1 has the highest affinity to glucose residues of the substrates among the five subsites in WBA and this subsite has a vital role in its catalytic activity (*31*). The crystal structures of maltose binding sites in SBA (*2*) and in BacBA (*79*) were also reported. Understanding the subsite structure of enzymes helps to predict the binding modes of substrates (*13*), and a glutamate residue was identified as the possible catalytic residue of soybean and sweet potato BAs (*80*, *81*).

The inhibitory effects of glucose (*13*), maltose (*13, 82*), and cyclohexa-amylose (*54, 55*) on BAs were studied. We described the inhibitory effects of maltose, glucose, and sugar derivatives on the catalytic activity of WBA in Chapter 2. We also described the activation and thermo-stabilization effects of additives on WBA and indicated the possibility of altering the stability and activity by modification of the enzyme reaction system in Chapter 1.

Temperature has substantial effect on the molecular activity as well as conformation of enzymes. The K_i values of inhibitors were affected by changes in temperature (*11, 12*). pH alters the ionization of the functional groups and conformation of enzymes and hence might affect the substrate or inhibitor binding. The effects of temperature and pH on the K_i and thermodynamic parameters of maltose and glucose inhibitions were described previously.

The fluorescence change of enzymes can be a good probe for examining the binding of substrates or inhibitors (*14*). The states of tryptophan and tyrosine residues of BacBA were affected up on binding maltose or glucose (*2, 13, 79*). Gluconolactone and maltose were reported to quench the fluorescence of glucoamylase (*83*). In the present study, we describe the WBA fluorescence quenching effects of maltose and glucose, the temperature- and pH-dependences of the association constant (K_a) of the association of WBA with the inhibitors, and thermodynamic parameters. This study provides valuable information on the interaction of the end-products of starch hydrolysis with WBA and its effect on the tryptophan and tyrosine residues. The changes in the states of tryptophan and tyrosine residues of WBA may be associated with the change in its activity by the interaction with maltose or glucose.

Materials and Methods

Materials - A commercial preparation of WBA, Himaltosin GS (Lot 2S24A), was purchased from HBI Enzymes (Osaka, Japan). WBA was purified from the Himaltosin preparation according to the method described in Chapter 1. Maltose (Lot M1B6462),

glucose (Lot M3G8543), and *N*-acetyl-L-tryptophan-ethyl ester (AWEE, Lot V6P4299) were purchased from Nacalai Tesque (Kyoto, Japan), *N*-acetyl-L-tyrosine-ethyl ester (AYEE, Lot 41666/1 42901) was from Fluka Chemicals (Buchs SG, Switzerland) and other chemicals were from Wako Pure Chemical (Osaka, Japan).

Fluorometric titration of WBA with maltose and glucose - WBA in buffer A was filtered through a Millipore membrane filter (Type HA; pore size: 0.45 μm) and twice through a Sephadex G-25 column equilibrated with the same buffer. The initial concentration of WBA was adjusted to 0.2 μM. Various initial concentrations of maltose (0-1.4 M) and glucose (0-2.8 M) were also prepared in buffer A. The fluorescence titration of the enzyme with increasing concentrations of maltose and glucose was carried out using a Shimadzu RF-5300PC spectrofluorometer (Kyoto, Japan) at 25°C, at an excitation wavelength 280 nm (λ_{ex} = 280 nm), with a high sensitivity and a response time of 4 s. The solvent perturbation effect on tryptophan and tyrosine was examined using 5.5 μM AWEE and 7.4 μM AYEE in the presence and absence of various concentrations of maltose or glucose.

The tryptophan fluorescence of WBA - The contributions of tryptophan and tyrosine residues in the fluorescence emission of WBA and how the fluorescence of tryptophan is quenched by maltose and glucose were studied. WBA (0.2 μM) was prepared in buffer A at 25^{o}C. The fluorescence spectra was collected in the range of 308-450 nm at λ_{ex} = 280 nm and at λ_{ex} = 295 nm, with a high sensitivity and a response time of 4 s. The fluorescence intensity of WBA in the absence and presence of high

initial concentrations of maltose (1.4 M) and glucose (2.8 M) at λ_{ex} = 295 nm were examined.

Temperature-Dependence of K_d - Various initial concentrations of maltose (0-1.4 M) and glucose (0-2.8 M) were prepared in buffer A at 25°C. The inhibitors were kept in water bath adjusted at each temperature and the enzyme solution was kept in ice water. The enzyme solution was incubated in a water bath at each temperature for 3 min and mixed in a cuvette with the inhibitors, and the fluorescence spectra were collected after 2 min at the same temperature. Only low concentrations (0-1 M) of both maltose and glucose were considered to determine the K_d values at 35 and 45°C, pH 5.4. The hyperbolic relationship of the change in fluorescence (ΔF) against the inhibitor concentration was converted to linear correlation so that the experimental data were fitted to linear plot using the least-squares regression method following previous report (*83*). The K_d values at each temperature were estimated using Hanes-Woolf plots (*62*, *83*). The standard enthalpy changes (ΔH^o) of maltose and glucose binding to WBA were determined by the van't Hoff equation (Eq. 1).

$$\ln K_d = (\Delta H^o/R)\,(1/T) - \Delta S^o/R \qquad (1)$$

The Gibbs energy change (ΔG^o) and entropy change (ΔS^o) were derived from Eqs. 2 and 3 (*59*, *60*).

$$\Delta G^o = -RT \ln K_d \qquad (2)$$

$$T\Delta S^o = \Delta H^o - \Delta G^o \qquad (3)$$

where R is the gas constant and T is temperature in kelvin.

pH-Dependence of K_d - The WBA and inhibitors (0-1 M) solutions were prepared in various buffers at 25°C, namely buffer A, 20 mM glycine-HCl at pH 3.0, and 20 mM borate buffer at pH 9.0. The procedure aforementioned was followed to obtain the K_d values at various pHs.

Results

Quenching the fluorescence of WBA by maltose and glucose - The fluorescence of WBA was partially quenched by increasing concentrations of maltose and glucose at 25°C, pH 5.4. The changes in the maximum fluorescence (ΔF_{max}) of WBA were 13% and 15% of the fluorescence intensity observed in the absence of inhibitor, respectively by the addition of 1.4 M maltose and by the addition of 2.8 M glucose (Fig. 1). The effects of increasing concentrations of maltose and glucose on the fluorescence of tryptophan and tyrosine were examined by fluorescence titration of model compounds, AWEE and AYEE. The net effects of increasing concentrations of the sugars were negligible (<2%) in AWEE and <4% in AYEE. The decrease in the fluorescence intensity of WBA by the titration with increasing maltose or glucose concentrations shows that the fluorescence of WBA is quenched by the interaction with maltose or glucose.

Quenching the fluorescence of WBA by concentrations of maltose up to 1.4 M and glucose up to 2.8 M were examined at 25°C, pH 5.4. The change in fluorescence (ΔF) is the difference between the fluorescence intensity (*FI*) of WBA in the absence of inhibitor (F_{max} WBA + buffer) minus the *FI* of WBA in the presence of inhibitors (F_{max}

WBA + inhibitors). It shows the change in fluorescence of WBA due to the interaction with increasing concentrations of maltose or glucose. The Michaelis-Menten-type hyperbolic relationships of ΔF *vs.* $[I]_o$ plots were shown (Fig. 2A). The fluorescence of WBA was partially quenched in a dose-dependent manner with increasing concentrations of maltose or glucose.

The plots of ΔF against lower inhibitor concentrations (0-1 M) obey a Michaelis-Menten-type relationship at all temperatures and pHs examined. Thus, this relationship was treated using the following equilibrium based on previous report (*83*):

$$EI \rightleftharpoons E+I \qquad (4)$$

where E, I, and EI represent the enzyme, inhibitor, and enzyme-inhibitor complex, respectively. In conditions where the enzyme concentration [E] is negligible compared to the inhibitor concentrations $[I]_o$, it can be written as:

$$[EI]_o = [E]_t[I]_o/(K_d+[I]_o) \qquad (5)$$

where K_d is the dissociation constant of the EI complex and $[E]_t$ is the total enzyme concentration. By considering that ΔF is proportional to [EI] (*83*), it can be rewritten in a linear form:

$$[I]_o/\Delta F = (K_d/\Delta F_{max}) + ([I]_o/\Delta F_{max}) \qquad (6)$$

where ΔF_{max} is the maximum decrease in fluorescence observed when the enzyme is saturated by maltose or glucose. The validity of equation (5) is confirmed by the linearity of the Hanes-Woolf plot ($[I]_o/\Delta F$ *vs.* $[I]_o$ plot) (Fig. 2B). The Hanes-Woolf plot for maltose concentration range (0-1.4 M) up to its higher concentration resulted in a higher K_d value (0.5 ± 0.6 M) with a larger standard deviation (SD) by considering that the binding of WBA and maltose was estimated by Eq. (4). We considered the possibility that the binding is composed of two modes with two K_d (K_{d1} and K_{d2}) values

(Fig. 2B). The lower concentration (0-1 M) of maltose showed a smaller K_d value (0.2 ± 0.1 M), which is similar to the K_i value, with a smaller SD. The binding of this lower concentration (0-1 M) of maltose is corresponding to the inhibitory binding to the active site of WBA. We showed the inhibition of WBA activity by lower concentrations of maltose (0-0.2 M) and glucose (0-0.5 M) in Chapter 2. The higher maltose concentration (1.1-1.4 M) gave a higher K_d value (1.5 ± 0.4 M) (Fig. 2B), suggesting secondary binding to WBA. Assuming that $K_{d2} >> K_{d1}$ and $[I] >> K_{d1}$, K_{d2} can be computed using the following equation.

$$[I]_o/\Delta F = [I]_o (K_{d2} + [I]_o)/(\Delta F_1 K_{d2} + (\Delta F_1 + \Delta F_2)[I]_o) \quad (7)$$

This indicates that the extrapolation of the straight line at high concentrations of $[I]_o$ will intersect the abscissa at $[I]_o = -K_{d2}$. The dotted lines in Fig. 2B were fitted to the data obtained using the concentrations of maltose >1 M. In the case of glucose, all the evaluated concentrations exhibited good regression ($R^2 = 0.99$) from the single linear fitting with the K_d value of 0.3 ± 0.1 M, which is in good agreement with the K_i values. Maltose binds to the active site as a competitive inhibitor in the range of 0-1 M and binds to the secondary binding site of WBA in the range of 1.1-1.4 M. Glucose binds to the active site of WBA as a competitive inhibitor in the range of 0-2.8 M.

Tryptophan fluorescence of WBA - The florescence emission of WBA due to its tryptophan and tyrosine residues was examined by changing the excitation wavelength from 280 nm to 295 nm (Fig. 3A). The fluorescence of WBA at λ_{ex} = 295 nm was quenched up to 8% by 1.4 M maltose and 9% by 2.8 M glucose (Fig. 3B). The Michaelis-Menten-type plots (Fig. 4A) are the ΔF of WBA observed by fluorescence titration of WBA with maltose and glucose at λ_{ex} = 295 nm. The K_d values of maltose

(0.3 ± 0.0 M) and glucose (0.5 ± 0.1 M) in quenching the tryptophan fluorescence were determined at 25°C, pH 5.4 (Fig. 4B). The ΔF at λ_{ex} = 295 nm with increasing concentrations of maltose and glucose indicates that the fluorescence of WBA due to its tryptophan residues was quenched by the interaction of WBA with maltose and glucose. It may be due to the change in the states of tryptophan residues of WBA to less hydrophobic conditions by the interaction with maltose and glucose.

Temperature-Dependence of K_d - The fluorescence of WBA reduced by about 20% with increasing temperature from 25 to 45°C. The respective K_d values of the WBA-maltose and WBA-glucose dissociations of lower concentrations (0-1 M) of maltose or glucose were estimated from the Hanes-Woolf plots at 35, and 45°C, at pH 5.4 (Fig. 5). These plots were derived from the ΔF of WBA with increasing concentrations of maltose or glucose according to Eq. 5. The K_d values of the WBA-maltose or WBA-glucose dissociations increased slightly with increasing temperature from 25 to 45°C (Table 1). The K_a values were 5.00 ± 1.12, 3.23 ± 0.84, and 2.70 ± 0.92 M^{-1} for maltose and 2.78 ± 0.85, 2.43 ± 0.91, and 2.17 ± 0.66 M^{-1}, respectively at 25, 35 and 45°C, at pH 5.4. Temperature is suggested to affect the molecular activity and the conformation of enzymes and hence it slightly affected the K_d and consequently the K_a values.

The thermodynamic parameters (ΔG^o and ΔS^o) of the binding of WBA with maltose or glucose were slightly affected by a change in temperature. The ΔG^o and K_a values were found to decrease in magnitude with increasing temperature (Table 1). The ΔG^o values were changed from -4.0 ± 0.8 to -2.5 ± 0.8 kJ mol^{-1} in the maltose binging and from -2.5 ± 0.9 to -1.9 ± 0.7 kJ mol^{-1} in the glucose binding to WBA by changing

temperature from 25 to 45°C, at pH 5.4. The ΔH^o values of the binding of WBA with maltose and glucose were determined from the van't Hoff plots (Fig. 6) to be -24.3 ± 3.2 kJ mol^{-1} and -9.7 ± 2.5 kJ mol^{-1}, respectively.

pH-Dependence of K_d - The effects of maltose and glucose on the fluorescence of WBA were studied at three different pHs (3.0, 5.4, and 9.0) at 25°C. The optimum pH for the WBA activity in starch hydrolysis is pH 5.4 (*36*). The relative activity of WBA in starch hydrolysis is about 60% at pH 3.5 and less than 10% at pH 7.5 relative to at its optimum pH 5.4 (*36*). The more acidic pH, 3.0 and alkaline pH, 9.0 conditions than the optimum pH, 5.4 were considered in this study. The K_d values were determined at each pH from the Hanes-Woolf plots as presented in Fig. 7A at pH 3.0 and Fig. 7B at pH 9.0. The Hanes-Woolf plot at pH 5.4, 25°C is shown in Fig. 2B. The estimated K_d values at pHs 3.0, 5.4, and 9.0 were almost the same as 0.2 ± 0.1 M for maltose and 0.3 ± 0.1 M for glucose. The K_d and ΔG^o values at various pHs were presented in Table 2. The pH of a reaction affects the ionizable groups of enzymes and the polarity of the solvent environment and hence, supposed to affect the interactions of enzymes with their inhibitors or substrates. However, the K_d values did not show considerable difference due to the pH change examined. The K_a values of WBA and maltose or glucose interactions and the ΔG^o values were not affected by changing the pH from 3.0 to 9.0 (Table 2).

Discussion

Quenching the fluorescence of WBA by maltose and glucose - The fluorescence of WBA was partially quenched by maltose and glucose. WBA (GenBank accession number X98504.1) has 10 tryptophan and 23 tyrosine residues (*84*). The interaction of certain amino acid residues which quench the fluorescence of tryptophan residues of SBA were affected up on binding substrates or final products (*85*). The variation in magnitude of ΔF depending on the size of substrates shows the presence of at least two tryptophan residues in the active site of SBA (*85*). The states of tryptophan and tyrosine residues of SBA were changed on binding with cyclohexadextrin and maltose, producing characteristic difference spectra in the ultraviolet region (*86*). In the hydrophobic solvent environment, the fluorescence intensity of a protein increases and the intensity peak shifts to a shorter wavelength by up to 15 nm (*87*). Thus, the states of tryptophan and tyrosine residues of WBA are converted to less hydrophobic condition by the addition of maltose or glucose. Maltose and gluconolactone inhibited the fluorescence of glucoamylase (*83*). The positions of Thr330 and Cys331 were altered by 1.08 and 1.14 Å, respectively, by binding maltose and affected the environment of tryptophan residues around the active site of BacBA (*2*). In addition to the movement of the flexible loop, the side chains of Tyr164 flipped by 34° because of maltose binding to BacBA, and the maltose binding to its C-terminal starch-binding domain interacts with Trp449 and Trp495 (*79*). These lines of evidence clarify that the fluorescence of WBA could be affected by the interaction with maltose or glucose. Identifying the specific tryptophan or tyrosine residues behind the fluorescence quenching by the interaction enables better understanding the role of binding subsites of the enzyme.

Despite many similarities among plant BAs, microbial BAs differ considerably from plant BAs in the starch-binding domain located at their C-terminal (*3*). According

to National Center for Biotechnology Information (NCBI) GenBank database, there are molecular distinctions even among plant BAs in the number of amino acids, molecular mass and number of Trp and Tyr residues. Using NCBI Basic Local Alignment Search Tool (BLAST), the amino acid sequence similarity of WBA is 69% with SBA, 82% with BBA and only 30% with BacBA based on the principle reported (*88*). These BAs vary in k_{cat} and K_m in soluble starch hydrolysis under similar experimental temperature and pH conditions (*13, 89*). The K_i (5.8 ± 1.1 mM) of maltose in SBA inhibition is much smaller than that in WBA inhibition (0.2 ± 0.0 M) while that of glucose (320 ± 80 mM) in SBA inhibition is in agreement with that in WBA inhibition (0.4 ± 0.0 M) at pH 5.4, 25^{o}C (*13*). WBA is low in thermal stability as compared with SBA, BBA, and BacBA. The T_{50} are 50^{o}C for WBA in Chapter 1, 57 and 63^{o}C for BBA and SBA, respectively (*26*). Hence, it could not be possible to extrapolate the experimental result of one BA to another.

Maltose exhibited two distinct K_d values at lower (0-1 M) and higher concentrations (1.1-1.4 M), suggesting that it has two modes of binding WBA. It appeared to bind to the active site of WBA at the lower concentrations with K_d value similar to the K_i value previously reported in Chapter 2. This may be the inhibitory binding at the active site and secondary binding mode at higher (1.1-1.4 M) concentration with higher K_d value, which is supposed to be different from the active site of WBA. Glucose appeared to have single mode of biding WBA in this study. In the inhibitory binding at the active site of WBA by glucose and lower concentration of maltose, they are supposed to quench the fluorescence of tryptophan and tyrosine residues around the active sites of WBA. The most probable fluorescing residues of WBA quenched by the interaction are Trp53, Trp196, Trp299, Tyr187, and Tyr416 based

on the crystal structure of WBA from SWISS-MODEL (*90*) using BBA template, PDB2XFR. However, crystallographic study may be required to confirm this fact and the experimental errors cannot be neglected because of handling small ΔF in this study. The subsite affinities of WBA were determined (*31*) following the subsite theory (*77*). Glucose binds to subsites 1 and 4, whereas maltose binds to subsites 1 and 2, and possibly to subsites 4 and 5 in SBA (*81*). In BacBA, glucose binds to subsites 1 and 2 (*13*), and to subsites 1, 2 or 4 in BBA (*91*). WBA has similar subsite affinities to SBA (*31*). Maltose is known to have secondary binding site at about 30 Å far from the active site, and also another binding site at domain C in BacBA (*2, 79*). Therefore, the sugars may have multiple binding modes or binding sites on BAs. Hence, only low concentrations of the inhibitors were considered for the estimation of K_d values in this study also based on previous recommendation (*69*).

The tryptophan fluorescence of WBA - The fluorescence emission of WBA due to its tryptophan residues was partially quenched by the interaction of WBA with maltose or glucose. The K_d values of maltose (0.32 ± 0.04 M) and glucose (0.51 ± 0.11 M) in quenching the tryptophan fluorescence of WBA at 25^{o}C, pH 5.4 were almost similar to their respective K_d values 0.23 ± 0.07 M and 0.41 ± 0.08 M in quenching the total fluorescence of WBA. This may also imply that quenching the fluorescence of WBA by the interaction with maltose or glucose is mainly by affecting the conditions of its tryptophan residues.

Temperature-Dependence of K_d - The K_d values of WBA-maltose and WBA-glucose dissociations were slightly affected by temperature as it influences the

molecular activities of the solvent and reactants. It also affects the structure of the protein, which entails changes in enzyme-inhibitor binding or dissociation (*62, 69*). The K_d values in this study and the K_i values from our previous kinetic study in Chapter 2 are in good agreement. A slight increase in K_d values with temperature was observed (Table 1). Increasing K_i values with increasing temperature were also previously reported in various enzymes (*59, 60, 69*). The condition of the solvent environment plays a decisive role in the fluorescence intensity of proteins. An increase in UV light absorption (A_{292}) of SBA with increasing concentration of maltose was ascribed to nonspecific solvent perturbation effect (*86*).

Temperature exerted slight influence on the thermodynamic parameters of maltose and glucose binding to WBA (Table 1). The ΔG^o value decreased by 1.5 kJ mol^{-1} in maltose and 0.6 kJ mol^{-1} in glucose with increasing temperature from 25 to 45^{o}C due to an increase in the entropy changes with increasing temperature. The negative ΔH^o shows that the bindings of maltose or glucose to WBA are exothermic and the ΔG^o indicates that the binding processes are spontaneous (Table 1). The crystallographic study of the binding of maltose and glucose to the active site of BacBA revealed that it is mainly by forming hydrogen-bonds (*2*). Higher ΔG^o values were obtained in maltose binding to WBA than that of glucose (Table 1), which agrees with their structural compositions. One maltose molecule contains two glucose residues and binds to two subsites of WBA while one glucose molecule binds to only one subsite and hence there are more H-bonds in maltose than glucose in binding to WBA.

pH-Dependence of K_d - The K_d and ΔG^o values did not show considerable difference with change in pH. There was redshift in λ_{max} of WBA fluorescence from 338

nm at pH 5.4 to 343 nm at pH 9.0 and 344 nm at pH 3.0. This is because of an exposure of the fluorescing residues of the enzyme to the polar solvent environment. The isoelectric point (p*I*) of WBA is 5.8 (*75*), which is similar to that of SBA, 5.6 (*76*). This implies that at pH 3.0, WBA is positively charged, and both maltose and glucose bind the active site in this state. The inhibition type depends on the binding sites of inhibitors (*78*). However, the K_a and thus ΔG^o values were not dependent on pH change from 3.0 to 9.0.

In conclusion, in the enzymatic starch hydrolysis process, maltose and glucose are continuously produced. Studying the molecular interaction of these end-products and WBA is valuable to identify the reaction condition where the product inhibition is relatively low and seek possible mechanisms of reducing the end-product inhibition. The ΔF of WBA by maltose and glucose showed that the tryptophan and tyrosine environments around its binding sites are affected as the result of binding the sugars. Quenching the fluorescence of WBA with maltose or glucose may be mainly by affecting the states of tryptophan residues. Maltose is suggested to have two binding modes to WBA at low and high concentrations. The K_a and ΔG^o values were slightly affected by temperature but not pH-dependent.

Table 1. The temperature-dependence of K_a and thermodynamic parameters of maltose and glucose binding to WBA.

	Temperature (K)	K_d (M)	K_i (M)	K_a (M^{-1})	ΔG^o (kJ mol^{-1})	$T\Delta S^o$ (kJ mol^{-1})
Maltose	298	0.20 ± 0.12	0.12 ± 0.02	5.00 ± 1.12	-3.99 ± 0.77	-20.33 ± 3.27
	308	0.31 ± 0.08	0.14 ± 0.04	3.23 ± 0.84	-2.90 ± 0.48	-21.42 ± 4.29
	318	0.37 ± 0.14	0.18 ± 0.04	2.70 ± 0.92	-2.46 ± 0.77	-21.86 ± 4.78
Glucose	298	0.36 ± 0.11	0.33 ± 0.03	2.78 ± 0.85	-2.53 ± 0.91	-7.18 ± 1.03
	308	0.41 ± 0.19	0.38 ± 0.03	2.43 ± 0.91	-2.21 ± 0.65	-7.50 ± 1.40
	318	0.46 ± 0.14	0.42 ± 0.04	2.17 ± 0.66	-1.92 ± 0.68	-7.79 ± 1.26

The experiment was conducted at various temperatures at pH 5.4, and the initial enzyme concentration $[E]_o$ was 0.2 μM. The ΔH^o values obtained from the van't Hoff plots were -24.3 ± 3.2 kJ mol^{-1} for maltose and -9.7 ± 2.5 kJ mol^{-1} for glucose binding to WBA. The values are mean ± SD. Each experiment was done in triplicates. The K_i values were from Chapter 2.

Table 2. The pH-dependence of K_a and ΔG^o of maltose and glucose binding to WBA.

	pH	K_d (M)	K_i (M)	K_a (M^{-1})	ΔG^o (kJ mol^{-1})
Maltose	3.0	0.22 ± 0.09	0.12 ± 0.04	4.55 ± 0.59	-3.75 ± 0.79
	5.4	0.20 ± 0.12	0.16 ± 0.03	5.00 ± 0.83	-3.99 ± 0.81
	9.0	0.17 ± 0.04	0.11 ± 0.04	5.88 ± 1.01	-4.39 ± 0.92
Glucose	3.0	0.29 ± 0.10	0.25 ± 0.03	3.45 ± 0.74	-3.07 ± 0.72
	5.4	0.36 ± 0.11	0.39 ± 0.03	2.78 ± 1.09	-2.53 ± 0.68
	9.0	0.26 ± 0.07	0.21 ± 0.03	3.85 ± 0.95	-3.34 ± 0.64

The experiment was conducted at various pHs at 25°C, and the initial enzyme concentration $[E]_o$ was 0.2 μM. The experiments were done in triplicates and the values are mean ± SD. The K_i values were from Chapter 2.

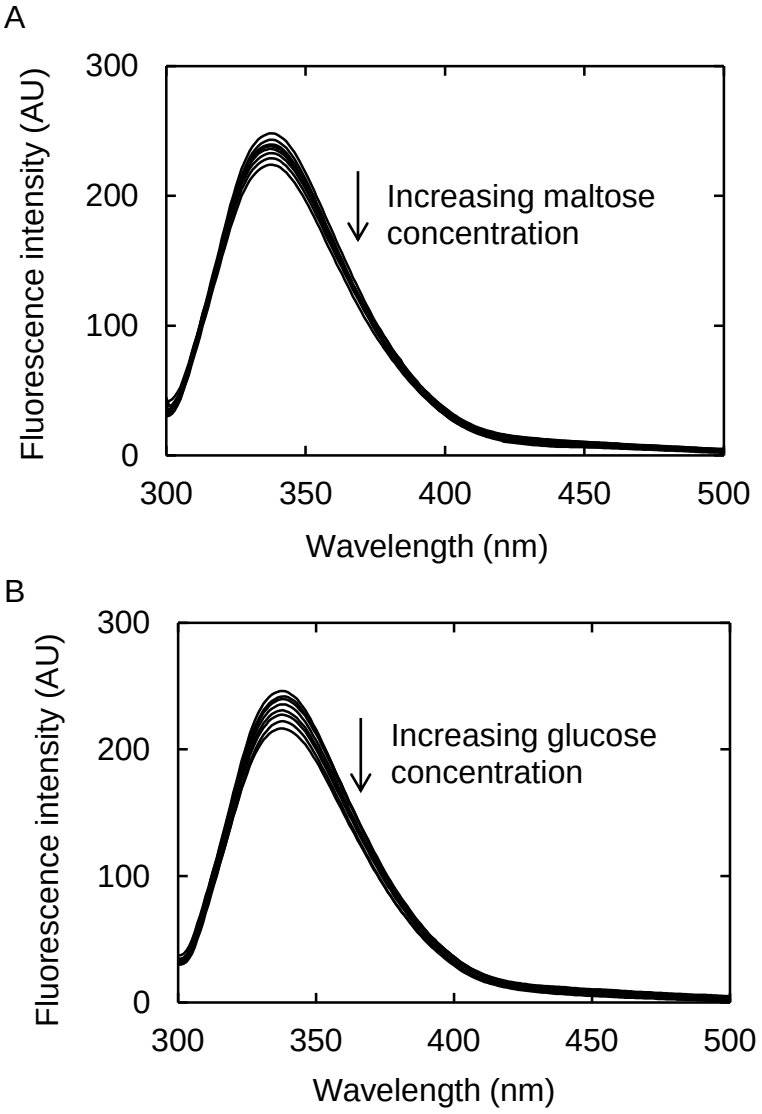

Fig. 1. Fluorometric titration of WBA by maltose and glucose. The effects of increasing concentrations of maltose 0-1.4 M (A) and glucose 0-2.8 M (B) on the fluorescence of WBA. The initial concentration of WBA was 0.2 μM. The fluorescence intensity of WBA was partially quenched up on titration with increasing concentrations of maltose or glucose at 25^{o}C, pH 5.4, and λ_{ex} = 280 nm.

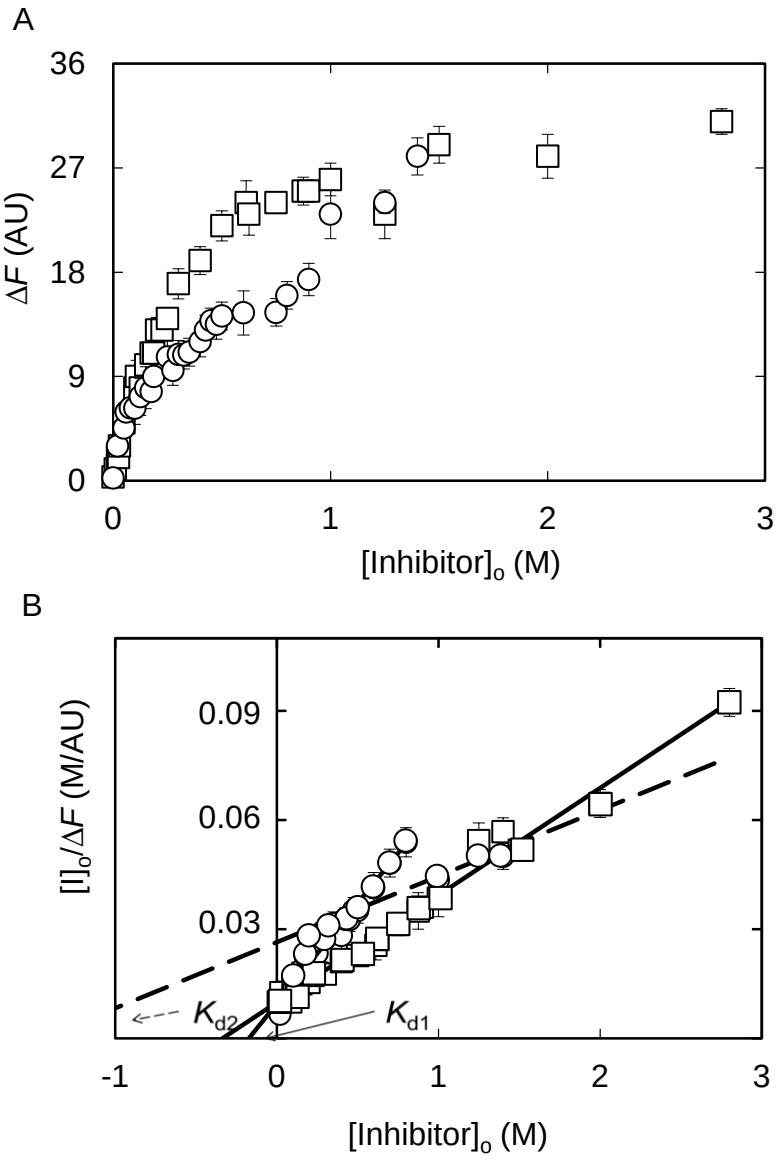

Fig. 2. Effects of maltose and glucose on the Δ*F* of WBA. The Δ*F* of WBA with increasing concentrations of maltose and glucose (A). Hanes-Woolf plots of the Δ*F* of WBA with increasing concentrations of maltose and glucose (B). The markers in both plots are for maltose (O) and for glucose (□). K_{d1} is the K_d value of maltose at lower concentration and K_{d2} at higher concentration. The experiment was conducted in triplicates at 25^{o}C, pH 5.4 at λ_{ex} = 280 nm.

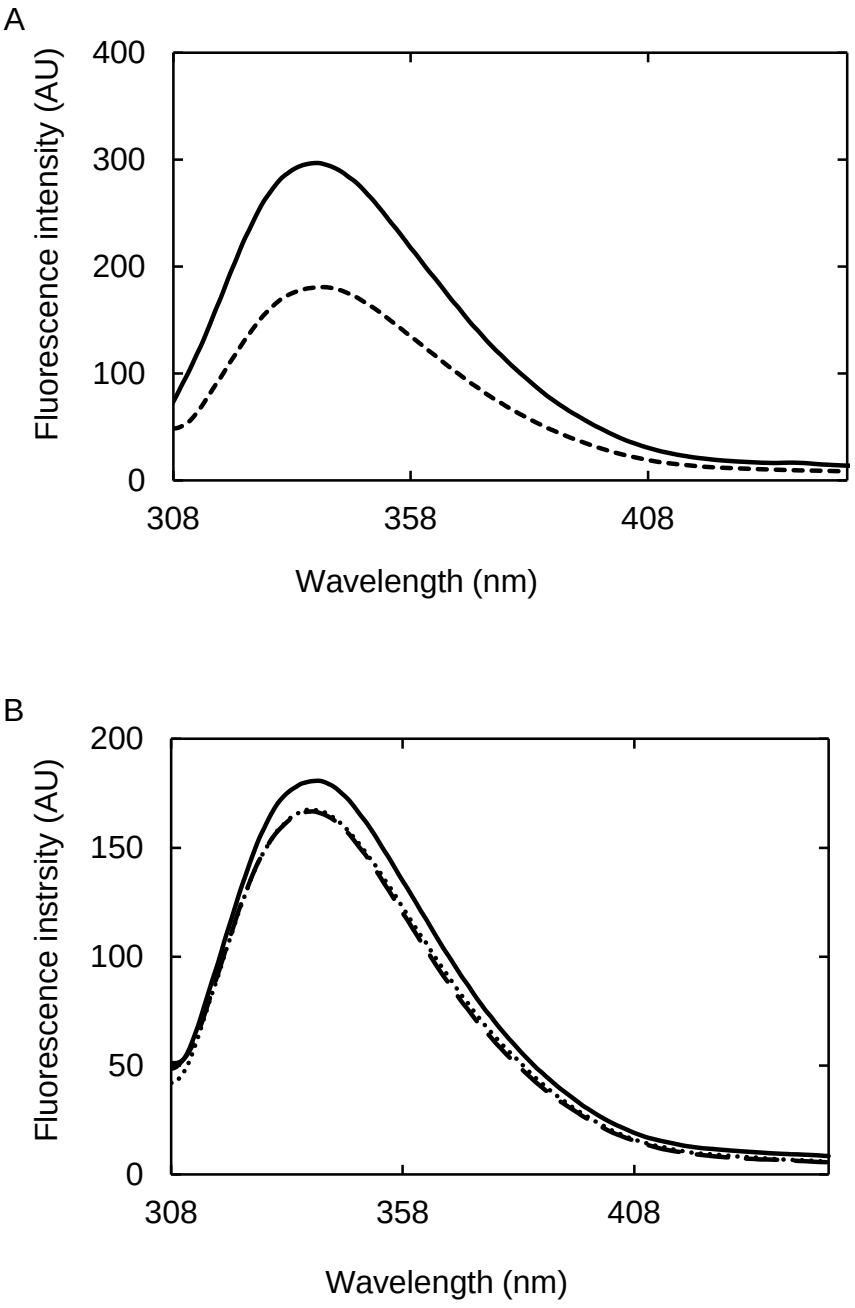

Fig. 3. Effects of maltose and glucose on the fluorescence intensity of WBA. The fluorescence of WBA with excitation at 280 nm (—) and at 295 nm (---) (A). The fluorescence of WBA excited at 295 nm in the absence of inhibitors (—), in the presence of 1.4 M maltose (⋯), and 2.8 M glucose (---) (B). The initial concentration of WBA was 0.2 μM and the experiment was conducted at 25^{o}C, pH 5.4.

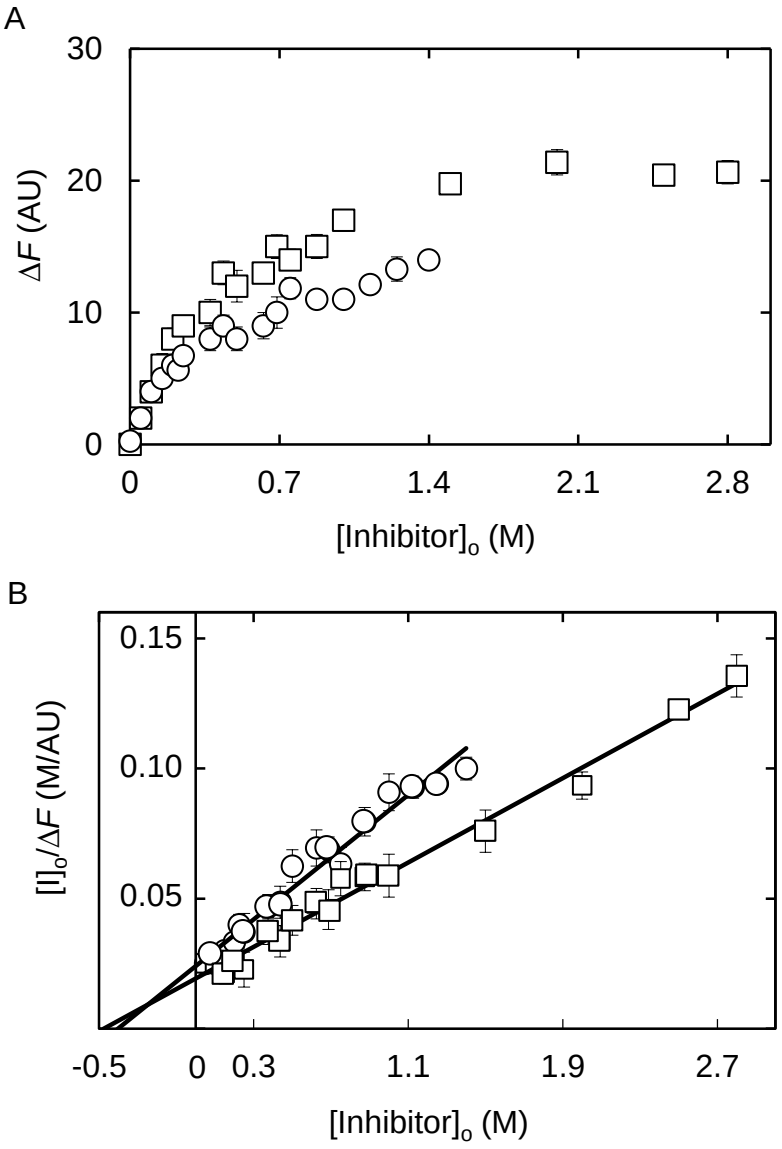

Fig. 4. Effects of maltose and glucose on the ΔF of WBA. The ΔF of WBA with increasing concentrations of maltose and glucose (A). Hanes-Woolf plots of ΔF with increasing concentrations of maltose and glucose (B). The markers in the plots are for maltose (O) and for glucose (□). The experiment was done in triplicates at 25^{o}C, pH 5.4, at λ_{ex} = 295 nm.

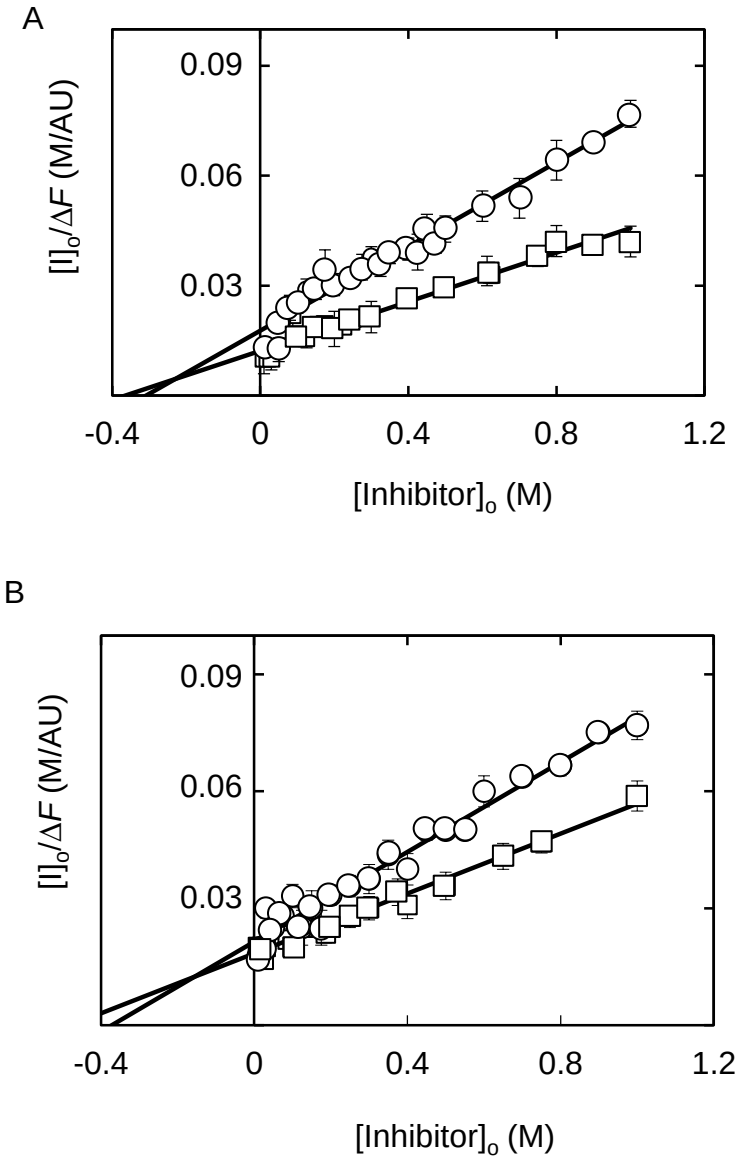

Fig. 5. Hanes-Woolf plots of ΔF with increasing concentrations of maltose and glucose. The experiment was done at 35°C (A) and 45°C (B), pH 5.4, and λ_{ex} = 280 nm. The Hanes-Woolf plot at 25°C, pH 5.4 was presented in Fig. 2B. The K_d values of maltose (O) and glucose (□) were obtained from the x-intercepts of the linear equations. The linear plots are independent for maltose and glucose under similar temperature and pH conditions; the graphs do not show type of inhibition.

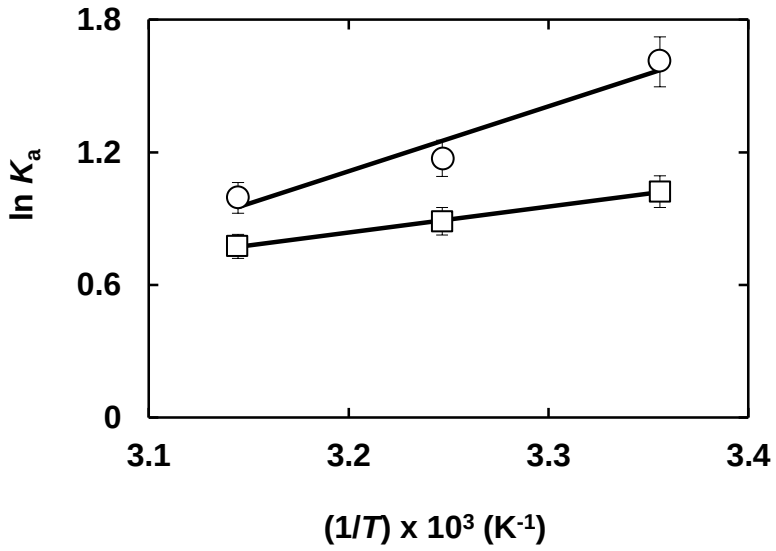

Fig. 6. van't Hoff plots of maltose and glucose quenching the fluorescence of WBA. The markers in the plots are for maltose (O) and for glucose (□). The ΔH^o of WBA binding maltose or glucose were obtained from the slope of the linear equations (slope = $-\Delta H^o/R$). The values of ΔH^o were -24.3 ± 3.2 kJ mol^{-1} for maltose and -9.7 ± 2.5 kJ mol^{-1} for glucose. The experiment was conducted at pH 5.4 and λ_{ex} = 280 nm.

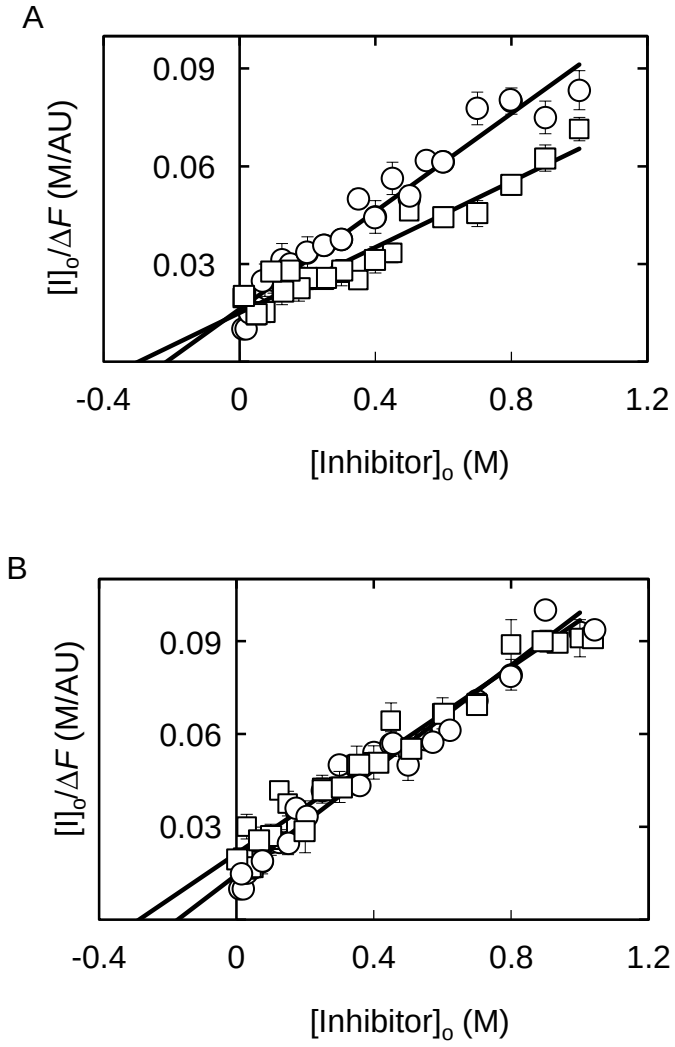

Fig. 7. The Hanes-Woolf plots of maltose and glucose quenching the fluorescence of WBA. The experiment was undertaken at pH 3.0 (A) and 9.0 (B) at 25^{o}C, and λ_{ex} = 280 nm. The Hanes-Woolf plot at pH 5.4, 25^{o}C was presented in Fig. 2B. The markers in the plots are for maltose (O) and for glucose (□). The x-intercepts of the linear equations give -K_d values in this study.

Chapter 4

Chemical Modification of Wheat β-Amylase by Trinitrobenzenesulfonic Acid, Methoxypolyethylene Glycol, and Glutaraldehyde to Improve Its Thermal Stability and Activity

Introduction

Enzymes can be stabilized through chemical modification, site-directed mutagenesis, immobilization, and solvent engineering (*6, 9, 10*). Nevertheless, selection of the appropriate techniques depends on various conditions (*23*). The thermal stability of enzymes has been improved by solvent modification with additives (*23, 24*). Sekiguchi *et al*. reported that bovine alkaline phosphatase was stabilized remarkably by amines and amino alcohols (*10*) and we have previously reported stabilization and activation of WBA by various additives such as ethanol, DMF, gelatin, glycine, and glycerol in Chapter 1.

WBA is a cheap alternative source of BA for industries. However, it is low in thermal stability compared to BAs from other crops and microbes. For instance, the T_{opt} of *Clostridium thermosulphurogenes* BA is 75^{o}C (*20*), the temperature at which BBA loses half of its activity after 30 min of incubation (T_{50}) is 57^{o}C and that of SBA is 63^{o}C (*26*) while the T_{opt} after 10 min incubation and T_{50} after 30 min incubation of WBA are 55 and 50^{o}C, respectively, in Chapter 1. Therefore, enhancing the activity and thermal stability of WBA is important for its industrial applications.

Chemical modification is one of the methods used in stabilizing enzymes and various chemicals were identified to modify specific residues of enzymes and confer better stability. Among which, TNBS, mPEG, and GA are known to make covalent interaction with the ε-amino groups of enzymes (*16-19*). The schematic illustrations of amino groups of protein modifications by mPEG (*19*) and TNBS (*92*) are shown in Fig. 1. GA is a bifunctional compound, which interacts with amino groups as shown in Fig. 1C or polymerized and forms interaction with protein (*93*).

GA covalently links to the amino groups of lysine or hydroxylysine in the protein molecules creating a structure more stable than that can be attained by the addition of salts, organic solvents, or non-ionic polymers (*16*). Modification of proteins with crosslinking agents like GA can be used for the reinforcement of the compact tertiary structures resulting in protein stabilization (*16*) and several chemical modifications have improved the thermal stability of proteases, like trypsin, chymotrypsin, and subtilisin (*94*). TNBS has been used for estimating the number of modified amino groups of proteins (*95-98*). It reacts with the amino groups of protein and makes stable complex (*96*). Polyethylene glycol (PEG) and its methoxy derivative, mPEG are additives largely employed in the pharmaceutical industry (e.g., for the formulation of protein-based drug to extend its half-life in the blood stream) and in the biotechnological field (*99, 100*). This interaction often induces structural changes of the entire molecule or its functional groups (*17, 18*).

In this chapter, we describe the importance of covalent modification of the amino groups of WBA by TNBS, mPEG, and GA in improving its thermal stability and catalytic activity.

Materials and Methods

Materials - A commercial preparation of WBA, Himaltosin GS (Lot 2S24A) was purchased from HBI Enzymes (Osaka, Japan). WBA was purified from the Himaltosin preparation according to the methods described previously to homogeneity as described in Chapter 1. Soluble starch (Lot M7H1482), maltose (Lot M1B6462), GA (Lot M1P3844), and coloring reagent A (0.38 M Na_2CO_3, 1.8 mM $CuSO_4$, and 0.2 M glycine) in the neocuproine method were from Nacalai Tesque (Kyoto, Japan). TNBS (Lot ALN6692) was from Wako Pure Chemical (Osaka, Japan) and mPEG or activated PEG_2 (Lot 01789-3) was from Seikagaku (Tokyo, Japan). Neocuproine-HCl (2,9-dimethyl-1,10-phenanthroline, Lot 032K2533) a coloring reagent B in the neocuproine method was from Sigma (St. Louis, MO, USA).

Number of amino groups modified by TNBS, mPEG, and GA - WBA, TNBS, mPEG, and GA were prepared in 50 mM phosphate buffer, pH 8.0 at 25^{o}C (referred as buffer B hereinafter). Equal volume of 1.2 μM WBA was incubated with 5.0 mM TNBS, 4.8 μM mPEG, and 10.6 mM GA in buffer B for 30 min in a water bath at 25^{o}C. The solutions of the enzyme-modifiers were dialyzed against buffer A 100 times the volume of the mixtures for 3 h at 4^{o}C. After dialysis, they were further modified by 5 mM TNBS at 25^{o}C for 30 min and their UV absorption spectra were evaluated using a Shimadzu UV-2200, UV-VIS recording spectrophotometer (Kyoto, Japan). Then the number of amino groups modified by TNBS, mPEG, and GA were estimated from the difference in absorbance at 340 nm using molar absorption coefficient 14.0 mM^{-1} cm^{-1}

of NH_2-TNP based on previously reported method (*101*) using the following equation (Eq. 1).

$$A_{340} = \varepsilon n \qquad (1)$$

where, A is absorbance; ε is absorption coefficient (14 mM^{-1} cm^{-1}); and n is number of modified amino groups of WBA.

Modification of WBA by TNBS - The initial concentration of WBA was adjusted to 1.2 μM spectrophotometrically in buffer A using the absorbance value (A) of 1.40 ± 0.02 at 281 nm with a 1.0-cm light-path for 1.0 mg/ml of WBA solution as reported in Chapter 1. Various initial concentrations of TNBS (0, 2.5, 5.0, and 10 mM) were also prepared in buffer B based on a method previously described (*102*). The enzyme and various concentrations of TNBS were mixed to initiate the modification and incubated at 25°C for 30 min. The enzyme-TNBS mixture was dialyzed using a dialysis membrane (Lot STJ6431) (Wako Pure Chemical) against 100 times its volume of buffer A for 3 h by changing the buffer every hour to remove the unreacted TNBS. The modified WBA (100 μl) was incubated for 30 min at various temperatures (25, 35, 45, 55, and 60°C) followed by mixing with 450 μl of soluble starch (0.82%, w/v) in buffer A at 25°C for the estimation of T_{50}. For evaluation of the effect of TNBS modification on the activity of WBA, the starch hydrolysis activity assay was conducted without heat treatment at 25°C. The enzyme reaction was stopped by adding 100 μl of 0.1 N NaOH into 12 μl of the enzyme-reaction solution. The amount of reducing sugar produced was determined using neocuproine method (*29*). Coloring reagent A and B of 150 μl each were mixed with the stopped enzyme-reaction solution (112 μl). The mixture was boiled for 8 min and diluted with 750 μl of water after cooling in ice water. The activity was measured at

450 nm using a Beckman-Coulter DU 800 spectrophotometer (Batavia, IL, USA) following the previously described method (*30*). The starch hydrolysis activity was calibrated to the concentration (mM) of maltose produced per minute.

Modification of WBA by mPEG - Various initial concentrations of $[WBA:mPEG]_o$ (1:0, 1:1, and 1:4 molar ratio) or 0, 1.2, and 4.8 μM of mPEG were prepared in buffer A based on the previously described method (*17*). The activity and thermal stability of WBA were evaluated in the same manner as explained above in the modification by TNBS. The degree of the amino groups modification by mPEG was evaluated from the change in absorbance at 340 nm (A_{340}) after 30 min reaction with TNBS.

Modification of WBA by GA - WBA (1.2 μM) was modified by various initial concentrations of GA (0, 1.1, 4.4, and 11 mM) prepared in buffer B. The stabilization effect of GA concentration up to 11 mM was examined as in the previous report (*103*). All the activity and thermal stability assays were undertaken in the same way aforementioned.

Thermal inactivation of WBA - The best concentrations of the modifier chemicals were selected based on their T_{50} values. In all cases of WBA modifications by TNBS, mPEG, and GA, the enzyme activity was measured after zero, 10, 20, and 30 min of pre-incubation for the estimation of the k_{obs} of the thermal inactivation assuming pseudo-first order kinetics by plotting ln (v/v_o) against the heat-treatment time (t) (Eq. 2), where v is the initial reaction velocity of the enzyme with heat treatment at each incubation temperature and v_o is the activity without heat treatment at 25°C. The E_a for

thermal inactivation was determined from the Arrhenius plots Eq. 3 (*104*). The $\Delta G^{o\ddagger}$ for thermal inactivation was obtained according to Eq. 4. $\Delta H^{o\ddagger}$ and $\Delta S^{o\ddagger}$ for thermal inactivation were estimated from Eyring plots (*11*, *32*) based on Eqs. 4 and 5.

$$\ln (v/v_o) = -k_{obs}t \quad (2)$$

$$\ln k_{obs} = -(E_a/R)(1/T) \quad (3)$$

$$\Delta G^{o\ddagger} = -RT[\ln (hk_{obs}/k_BT)] \quad (4)$$

$$\ln (hk_{obs}/k_BT) = -(\Delta H^{o\ddagger}/RT) + (\Delta S^{o\ddagger}/R) \quad (5)$$

where, R is the gas constant; h is the Planck constant; k_B is the Boltzmann constant; and T is temperature in kelvin.

Results

Number of amino groups of WBA modified by TNBS, mPEG, and GA - The UV absorbance peak at A_{340} of WBA previously modified by mPEG and GA were reduced as compared to that of unmodified WBA (Fig. 2). In WBA modified by mPEG and GA, the decrease in A_{340} was because some of the amino groups were already modified by the chemicals and the amino groups modified by mPEG and GA do not have absorbance peak at 340 nm. The A_{340} values of WBA modified by 4.8 μM mPEG, 11 mM GA were reduced to 0.26, 0.22, respectively from 0.52 by 5 mM TNBS, thus 6 amino groups of WBA were modified by 5 mM TNBS while 4 amino groups of WBA previously modified by 4.8 μM mPEG and 5 amino groups were modified by 11 mM GA in 30 min incubation time.

The activity of WBA modified by TNBS, mPEG, and GA - The catalytic activities of WBA modified by different chemicals were compared with that of unmodified WBA at 25^{o}C, pH 5.4. The rate of soluble starch hydrolysis by WBA modified by 4.8 μM mPEG with 4 amino groups modified was enhanced by 39% while those of WBA modified by 5 mM TNBS and 11 mM GA were reduced by 15 and 59%, respectively, in comparison with that of unmodified WBA (Fig. 3A). The activities of WBA modified by TNBS, mPEG, and GA and the relationship between the number of amino groups modified by TNBS and GA and loss of catalytic activity are presented (Fig. 3B). The activities (%) of WBA modified by 1, 5, and 10 mM TNBS were 96, 85, and 40% with modification of 3, 6, and 8 amino groups, respectively. With modification of 2, 4, and 5 amino groups by 1.1, 4.4, and 10.6 mM GA, the respective relative activities were 87, 67, and 33%. The activity of WBA treated in buffer in the same manner as with the modifier chemicals was considered to be 100%. Modification by GA resulted in stabilization effect equivalent to that by mPEG but it reduced the activity of the enzyme. WBA modification by mPEG was found to have both activation and stabilization effects.

Thermal inactivation of WBA modified by TNBS, mPEG, and GA - The concentrations of TNBS, mPEG, and GA were considered in the thermal inactivation study based on their highest T_{50} values. The thermodynamic parameters (E_a, $\Delta G^{o\ddagger}$, $\Delta H^{o\ddagger}$, and $\Delta S^{\ddagger}$) for thermal inactivation of the modified and unmodified WBA were evaluated using Arrhenius plots (Fig. 4A) and Eyring plots (Fig. 4B). The E_a values were 44 ± 2, 32 ± 2, 26 ± 1, and 24 ± 2 kJ mol^{-1} for the unmodified, WBA modified by 5.0 mM TNBS, 4.8 μM mPEG, and 10.6 mM GA, respectively. The respective number of amino groups modified by TNBS, mPEG, and GA were 6, 4, and 5. The E_a of WBA modified

by TNBS, mPEG, and GA values were less than that of unmodified enzyme as shown in Table 1. The $\Delta S^{o\ddagger}$ and $\Delta H^{o\ddagger}$ values of modified WBA were reduced as compared with that of unmodified WBA (Table 1). In this study, the results suggest that the molecular interactions of these modifying chemicals with WBA are not similar, even though they were suggested to form covalent modification of the enzyme's amino groups.

Thermal stability of WBA modified by TNBS - The modification of WBA by TNBS was examined using the change in A_{340} with increasing modification time (Fig. 5A) and with increasing concentration of TNBS (Fig. 5B). The absorbance peak at 340 nm increased with modification time. The T_{50} values of WBA modified by various concentrations of TNBS were compared against that of unmodified WBA treated in the same manner in buffer A. The T_{50} values were 46 ± 2, 47 ± 1, and 48 ± 2^{o}C with TNBS concentration of 0.5, 5, and 10 mM (Fig. 6A) with modification of 3, 6, and, 8 amino groups (Fig. 7), respectively indicating that the modification has a little effect on the stability. The thermodynamic parameters for thermal inactivation of unmodified and modified WBA by TNBS, mPEG and GA are shown (Table 1). The E_a value of WBA with 6 amino groups modified by 5 mM TNBS was reduced to 32 ± 1 kJ mol^{-1} compared with 44 ± 2 kJ mol^{-1} of the unmodified WBA. The activities of WBA modified by various concentrations of TNBS thermally treated at 25^{o}C were considered as 100% and the relative activities after thermal treatment after 30 min at various temperatures were calculated relative to their respective activities at 25^{o}C. The k_{obs} value was determined from the thermal inactivation progress curve with increasing incubation time (0, 10, 20, and 30 min) at each incubation temperature from the plots of ln (v/v_o) *vs.* incubation time. The k_{obs} values of unmodified and modified WBA are

presented in Table 2.

Thermal stability of WBA modified by mPEG - The interaction of mPEG with proteins was illustrated in Fig. 1B. The thermal stability of WBA modified by mPEG showed substantial increase in its T_{50} after 30 min of pre-incubation at various temperatures (Fig. 6B). The T_{50} of WBA with 4 amino groups modified by 4.8 μM mPEG was increased from 47 ± 1 to 55 ± 2 by 8°C.

The E_a value of unmodified WBA was 44 ± 2 kJ mol^{-1} and that of WBA modified by 4.8 μM mPEG was 26 ± 2 kJ mol^{-1}. The $\Delta S^{o\ddagger}$ of WBA modified by mPEG was significantly reduced (Table 1). The activity loss of WBA modified by 4.8 μM mPEG with increasing heat treatment time was slower than that of unmodified enzyme especially at high temperature. The $\Delta H^{o\ddagger}$ and $\Delta S^{o\ddagger}$ values were reduced compared with that of unmodified WBA (Table 1).

Thermal stability of WBA modified by GA - The stability of WBA was significantly improved by modification with 11 mM GA increasing its T_{50} from 47 ± 1 to 54 ± 2 by 7°C with 5 amino groups modified (Fig. 6C and Fig. 7). The rate of activity loss with increasing incubation time of the modified WBA by GA particularly at high temperature was slower than that of unmodified enzyme and the k_{obs} are shown in Table 2.

Discussion

Number of amino groups of WBA modified by TNBS, mPEG, and GA - The A_{340} of WBA previously modified by mPEG and GA were reduced as compared to that of unmodified WBA (Fig. 2). The modification of amino groups of the enzyme molecule was evaluated spectrophotometrically by the change of absorbance at 340 nm, derived from the N^{ε}-tri-nitrophenyl group of the lysine residue (*101*). The reaction between the amino groups and TNBS gives orange colored product, which has absorbance peak around 340 nm and 420 nm, and it helps to monitor the modification of amino groups of proteins. According NCBI GenBank database, WBA (GenBank accession number X98504.1) has 32 Lys residues (*84*). The number of amino groups modified by mPEG and GA can be estimated from the decrease in A_{340} (*16, 98, 101, 105*). PEG conjugates with the amino groups of lysine residues and N-terminal of proteins, and lysine is the most prevalent amino acid residue in proteins, which can make more than 10% of the overall amino acid sequence (*106*). The amino groups of Cys, His, and N-terminal may also react with the chemical modifiers used in this study based on previous reports (*26, 106*).

The activity of WBA modified by TNBS, mPEG, and GA - The rate of soluble starch hydrolysis by WBA modified by 4.8 μM mPEG with 4 amino groups modified was enhanced while those of WBA modified by 5 mM TNBS and 11 mM GA were reduced in comparison with that of unmodified WBA (Fig. 3A). Enhancement of the catalytic activity of lipase up to 3.7-fold by mPEG was suggested to be by hindering intermolecular protein-protein interactions and increasing the accessibility and flexibility of the enzyme (*100*). An increase in the catalytic activity of L-asparaginase upon pegylation during lyophilization was reported (*107*). On the other hand, it was

reported that modification of lysine residues by TNBS has resulted in decreasing 50% of amylase activity, whereas maltosidase activity was enhanced to 250% (*101*). Incubation of 1,4-β-D-glucan glucanohydrolase with various concentrations of TNBS exhibited a time and concentration dependent loss of enzyme activity (*102*). Acid phosphomonoester hydrolase lost greater than 50% of its activity by GA (*108*).

Thermal inactivation of WBA modified by TNBS, mPEG, and GA - The E_a of WBA modified by TNBS, mPEG, and GA values were less than that of unmodified enzyme as shown in Table 1. This is because the rate of activity loss that gives the k_{obs} of the modified enzymes was slower compared with that of unmodified enzyme (Table 2). Kinetic stability depends on the energy barrier to unfolding (i.e., the activation energy [E_a] of unfolding) (*109*). The unfolding in this case is the partial unfolding in the transitional state, not the irreversible unfolding. The E_a between folded and transition-state decrease as a result of smaller k_{obs} and the stabilization is entropic-driven. The smaller $\Delta H^{o\ddagger}$ in the stabilized enzyme indicates that there is smaller energy difference between the folded and transitional states compared with that of unstabilized WBA. An increase of the transition enthalpy indicates formation of noncovalent bonds in the native structure (*110*). However, the enthalpy change of the transition state of WBA was reduced in this study, indicating no more formation of noncovalent bonds. The free energy of stabilization (ΔG_{stab}, where $\Delta G_{stab} = \Delta H_{stab} - T\Delta S_{stab}$) of a protein is the difference between the free energies of the folded and the unfolded states of that protein and it directly measures the thermodynamic stability of the folded protein (*109*). Stability studies of mutants showing differences in ΔG_{stab} as small as 3.0-6.5 kJ mol^{-1} can account for stability increase of up to 12^{o}C (*109*). An increase in the stability of

modified Taka-amylase (TAA_{MOD}) relative to unmodified Taka-amylase (TAA_{UM}) was accompanied by a decrease in the values of $\Delta H^{o\ddagger}$ and $\Delta S^{o\ddagger}$ from the Arrhenius and Eyring plots (*111*). In the transitional state, the protein is partially unfolded, and the previously buried hydrophobic residues are exposed to the solvent. The exposed hydrophobic residues may cause an ordering of the water molecules around them, thereby reducing the entropy of the transitional state (*110*). It was also proposed that proteins of known three-dimensional structure could be stabilized by decreasing their entropy of unfolding and stable mutants have smaller $\Delta H^{o\ddagger}$ and $\Delta S^{o\ddagger}$ values relative to the native, less stable one (*112*). The decrease in $\Delta S^{o\ddagger}$ may suggest that the transition-state may have ordered water molecules on the hydrophobic parts of the modified WBA. The advantage of modified over unmodified enzymes can be illustrated by the productivity assay at a certain temperature over a course of time (*111*).

Thermal stability of WBA modified by TNBS - The modification of WBA by TNBS was examined using the change in A_{340} with increasing modification time (Fig. 5A) and with increasing concentration of TNBS (Fig. 5B). This confirms that the amino groups of WBA were modified by TNBS. However, WBA modification by TNBS had very slight thermal stabilization effect.

Thermal stability of WBA modified by mPEG - The thermal stability of WBA modified by mPEG showed substantial increase in its T_{50} after 30 min of pre-incubation at various temperatures (Fig. 6B). This level of thermal stability improvement is not small in view of the stabilization attained through solvent modification by various additives in our previous study in Chapter 1. Chemical modification of proteins by PEG

has been extensively studied in biomedical and biotechnological processes for the reduction of their immunogenicity and improving the life of pharmaceutical proteins in blood (*113*). The PEG-L-asparaginase *in vitro* assays showed high stability in human serum sample, keeping its activity practically unchanged for 40 min, showing improved resistance against proteases in the serum (*37*). Chemical modification of the amino groups of lipase by mPEG has improved its thermal stability (*113*). Pegylation of WBA improved its T_{50} from 47 ± 1 to 55 ± 2^{o}C, which is comparable to that of BBA (*26*).

Thermal stability of WBA modified by GA - The stability of WBA was significantly improved by modification with 11 mM GA increasing its T_{50} by 7^{o}C with 5 amino groups modified (Fig. 6C and Fig. 7). The stabilization of protein molecules by GA was reported (*114*). Treatment of collagen fibers by aldehydes increased its hydrothermal shrinkage temperature from 62 to 77-86^{o}C (*103*). The treatment of glucose oxidase (GOX) with GA yielded higher stabilization (*114*). These reports show the possibility of stabilizing enzymes by modification with GA. The $\Delta S^{o\ddagger}$ values of the modified WBA were reduced comparatively indicating structural or conformational stability.

In conclusion, chemical modification of WBA by mPEG and GA substantially improved its thermal stability. The modification by mPEG and GA affected its $\Delta S^{o\ddagger}$ rather than the $\Delta H^{o\ddagger}$ of thermal inactivation. WBA modification by mPEG improved both its activity and stability. The thermal stability of WBA was considerably improved by modification with GA but lost its catalytic activity.

Table 1. Thermodynamic parameters of the thermal inactivation of unmodified and modified WBA by TNBS, mPEG, and GA.

Temperature (^{o}C)	Control (Buffer)			TNBS (5 mM)			mPEG (4.8 μM)			GA (11 mM)		
	$\Delta G^{o\ddagger}$ (kJ mol^{-1})	$\Delta H^{o\ddagger}$ (kJ mol^{-1})	$T\Delta S^{o\ddagger}$ (kJ mol^{-1})	$\Delta G^{o\ddagger}$ (kJ mol^{-1})	$\Delta H^{o\ddagger}$ (kJ mol^{-1})	$T\Delta S^{o\ddagger}$ (kJ mol^{-1})	$\Delta G^{o\ddagger}$ (kJ mol^{-1})	$\Delta H^{o\ddagger}$ (kJ mol^{-1})	$T\Delta S^{o\ddagger}$ (kJ mol^{-1})	$\Delta G^{o\ddagger}$ (kJ mol^{-1})	$\Delta H^{o\ddagger}$ (kJ mol^{-1})	$T\Delta S^{o\ddagger}$ (kJ mol^{-1})
25	90 ± 3	42 ± 1	-48 ± 2	88 ± 1	29 ± 2	-59 ± 2	87 ± 2	23 ± 1	-64 ± 1	88 ± 2	21 ± 2	-67 ± 3
35	92 ± 2	42 ± 2	-50 ± 1	91 ± 3	30 ± 1	-61 ± 1	90 ± 1	24 ± 2	-66 ± 2	91 ± 2	22 ± 1	-69 ± 2
45	94 ± 2	43 ± 2	-51 ± 2	94 ± 2	31 ± 2	-63 ± 1	92 ± 1	24 ± 1	-68 ± 1	94 ± 1	22 ± 1	-72 ± 1
55	94 ± 3	41 ± 1	-53 ± 1	95 ± 2	30 ± 2	-65 ± 2	94 ± 2	24 ± 2	-70 ± 3	96 ± 2	22 ± 2	-74 ± 1

The enthalpy and entropy changes of thermal inactivation were obtained from Eyring plots. $\Delta H^{o\ddagger}$ = slope/R; and $\Delta S^{o\ddagger}$ = -R x y-intercept where R is the gas constant. The E_a values from the Arrhenius plots were 44 ± 2, 32 ± 2, 26 ± 1, and 24 ± 2 kJ mol^{-1} for the unmodified, WBA modified by 5.0 mM TNBS, 4.8 μM mPEG, and 11 mM GA, respectively with modification of 6 amino groups by TNBS, and 4 amino groups by mPEG and 5 amino groups by GA. The experiments were done at various temperatures and pH, 5.4. The initial concentration of enzyme was 1.2 μM and soluble starch was 0.82% (w/v). The smaller E_a and $\Delta H^{o\ddagger}$ values in the stabilized enzyme were because of slower progressive loss of activity (smaller k_{obs}) with increasing temperature and it shows smaller energy difference between the unfolded and folded states compared to that of unstabilized enzyme and the stabilization is mainly by reducing the $\Delta S^{o\ddagger}$.

Table 2. The k_{obs} (min^{-1}) of WBA modified by TNBS, mPEG, and GA.

Temperature (^oC)	Buffer (no amino group modified)	5 mM TNBS (6 amino groups modified)	5 μM mPEG (4 amino groups modified)	11 mM GA (5 amino groups modified)
	k_{obs} x 10^3 (min^{-1})			
25	2 ± 1 (100)	2 ± 0 (100)	2 ± 1 (100)	1 ± 1 (50)
35	6 ± 2 (300)	5 ± 1 (250)	4 ± 1 (200)	2 ± 1 (100)
45	11 ± 2 (550)	7 ± 0 (350)	6 ± 2 (300)	3 ± 1 (150)
55	14 ± 2 (700)	9 ± 1 (450)	8 ± 1 (400)	5 ± 2 (250)

The concentration of WBA was 1.2 μM and initial substrate concentration was 0.82% (w/v) in buffer B. The temperature for both modification and hydrolysis was 25^{o}C and the temperature in the table is the incubation temperature for 30 min. The values of k_{obs} were obtained from the ln (v/v_o) *vs.* incubation times (0, 10, 20, and 30 min) plots. The numbers in the parenthesis show the values of k_{obs} (%) at various temperatures relative to that of at 25^{o}C. The k_{obs} of the unmodified WBA at 25^{o}C was considered to be 100%.

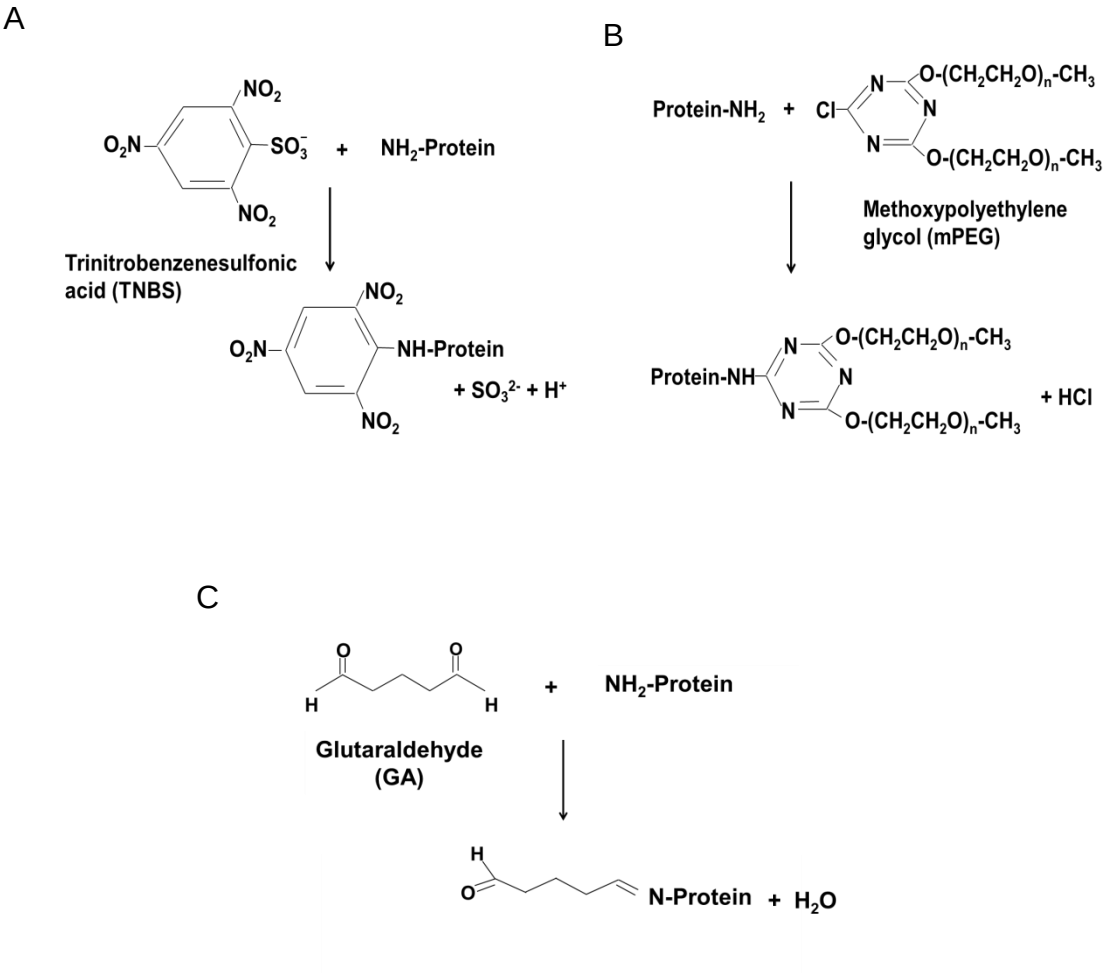

Fig. 1. The molecular structures of protein modification. Modification of protein amino groups by TNBS (A), mPEG (B), and GA (C). The scheme shows how the modifier chemicals make covalent interaction with the amino groups of proteins.

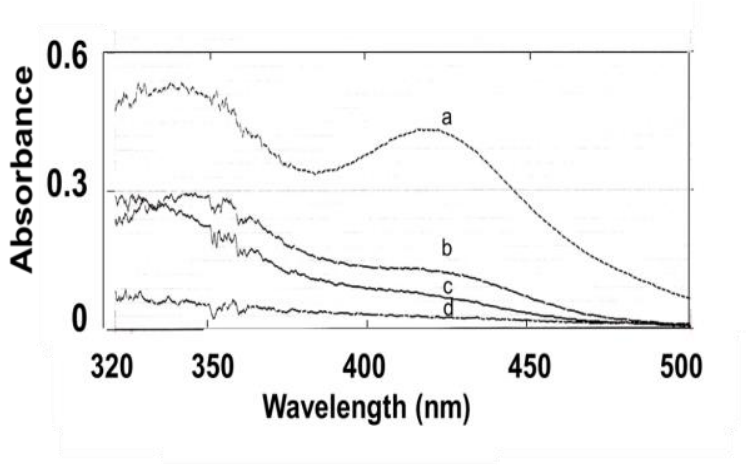

Fig. 2. The spectra of WBA modified by 5 mM TNBS for 30 min. The letters in the figure show the modifier chemicals with which WBA was previously modified before modification by TNBS: a, buffer; b, mPEG; c, GA; and d, buffer at zero incubation time. The number of amino groups of WBA modified by mPEG and GA were determined from the change in A_{340} with reference to that of unmodified WBA.

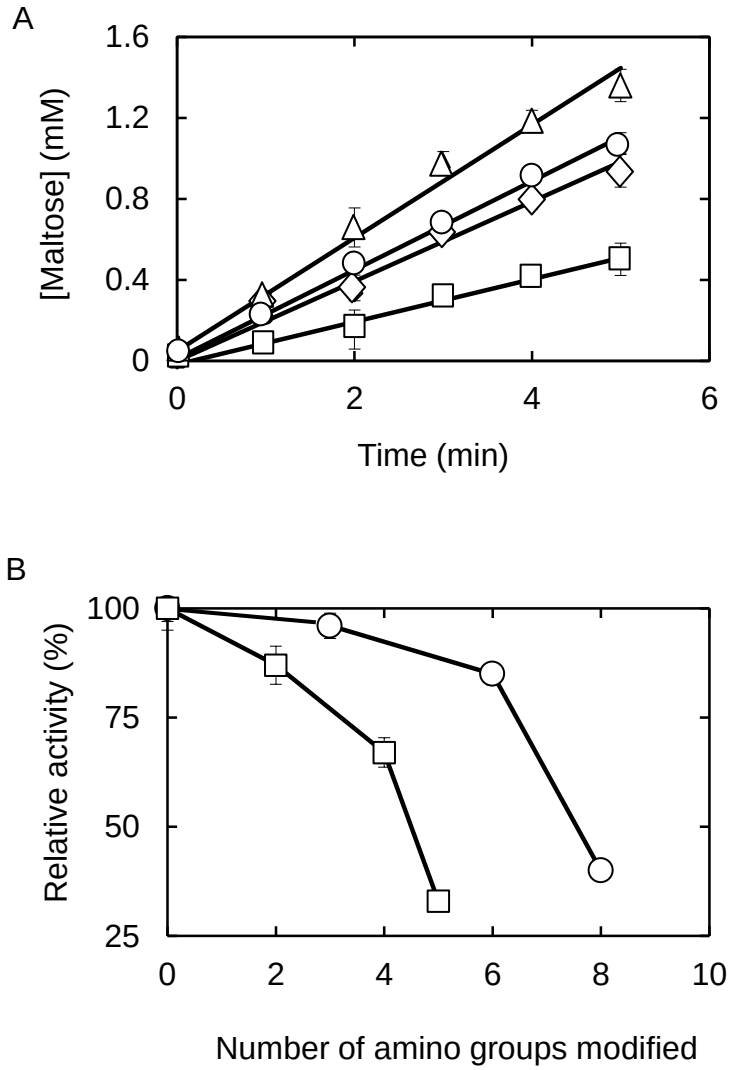

Fig. 3. The effects of WBA modification on its activity. The starch hydrolysis activities of unmodified WBA (O) and WBA modified by: 5.0 mM TNBS (□), 4.8 μM mPEG (Δ), and 10.6 mM GA (◇) (A). The relationship between the activity loss and the number of amino groups modified by TNBS (O) and GA (□) (B). The number of modified amino groups was determined as mentioned in the Materials and Methods. The catalytic activities were measured after 3 min pre-incubation time at 25^{o}C, pH 5.4.

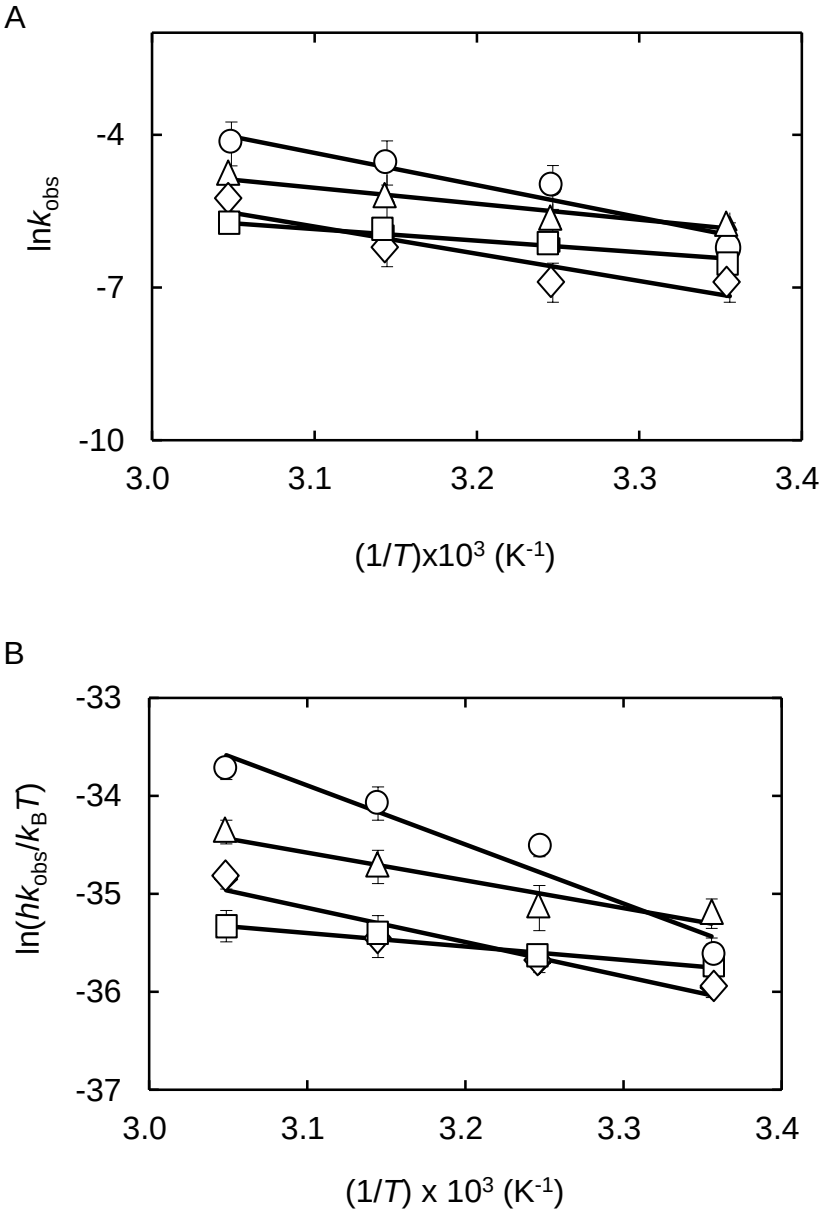

Fig. 4. Thermal inactivation of modified WBA. Arrhenius plots (A) and Eyring plots (B) for thermal inactivation of WBA modified by TNBS, mPEG, and GA. The markers in both plots indicate WBA modified by various chemicals: unmodified (O), 5.0 mM TNBS (◇), 4.8 μM mPEG (Δ), and 10.6 mM GA (□). The slopes of the Arrhenius plots give E_a= -R x slope. The slopes of the Eyring plots give $\Delta H^{o\ddagger}$ (slope= $-\Delta H^{o\ddagger}/R$) and y-intercept = $\Delta S^{o\ddagger}/R$, as explained in the Materials and Methods.

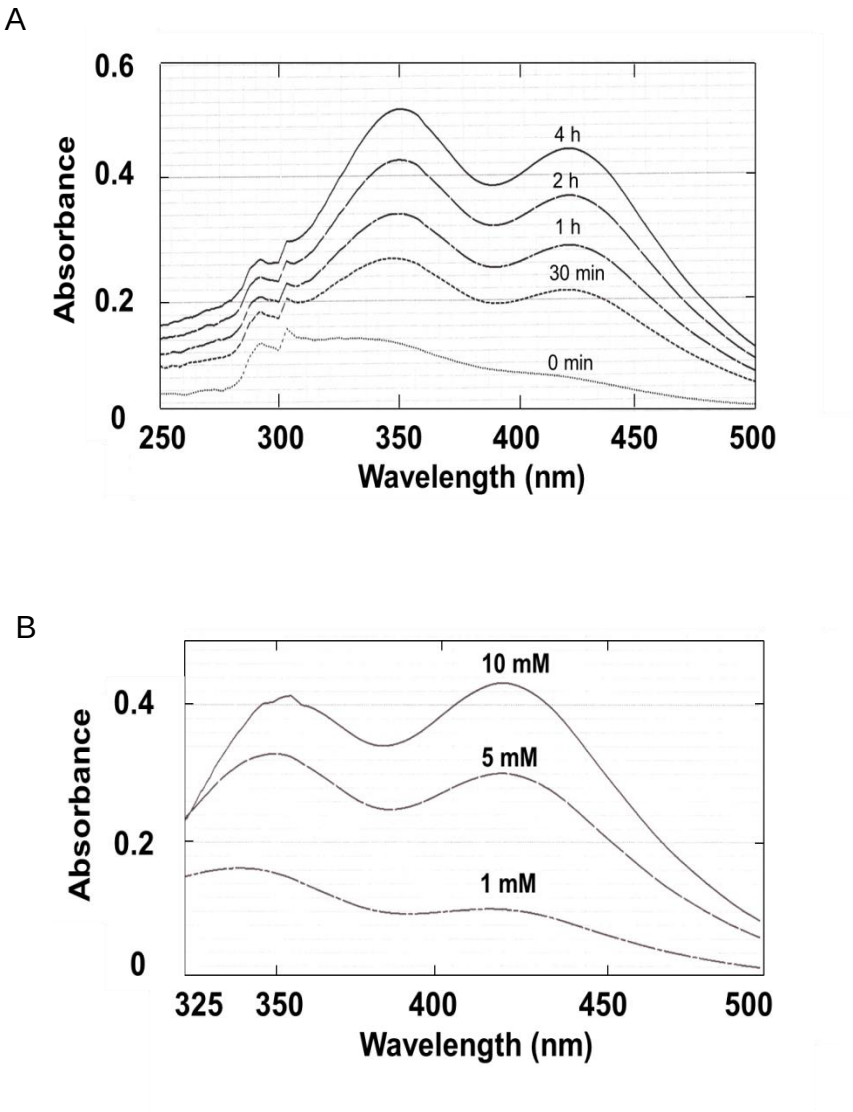

Fig. 5. The spectra of WBA modified by NBS. Modification of WBA by 5 mM TNBS at various incubation or modification durations (A). Modification of WBA by increasing TNBS concentration on the number of amino groups modified after 30 incubation time (B). The A_{340} of WBA increased with incubation time and TNBS concentration.

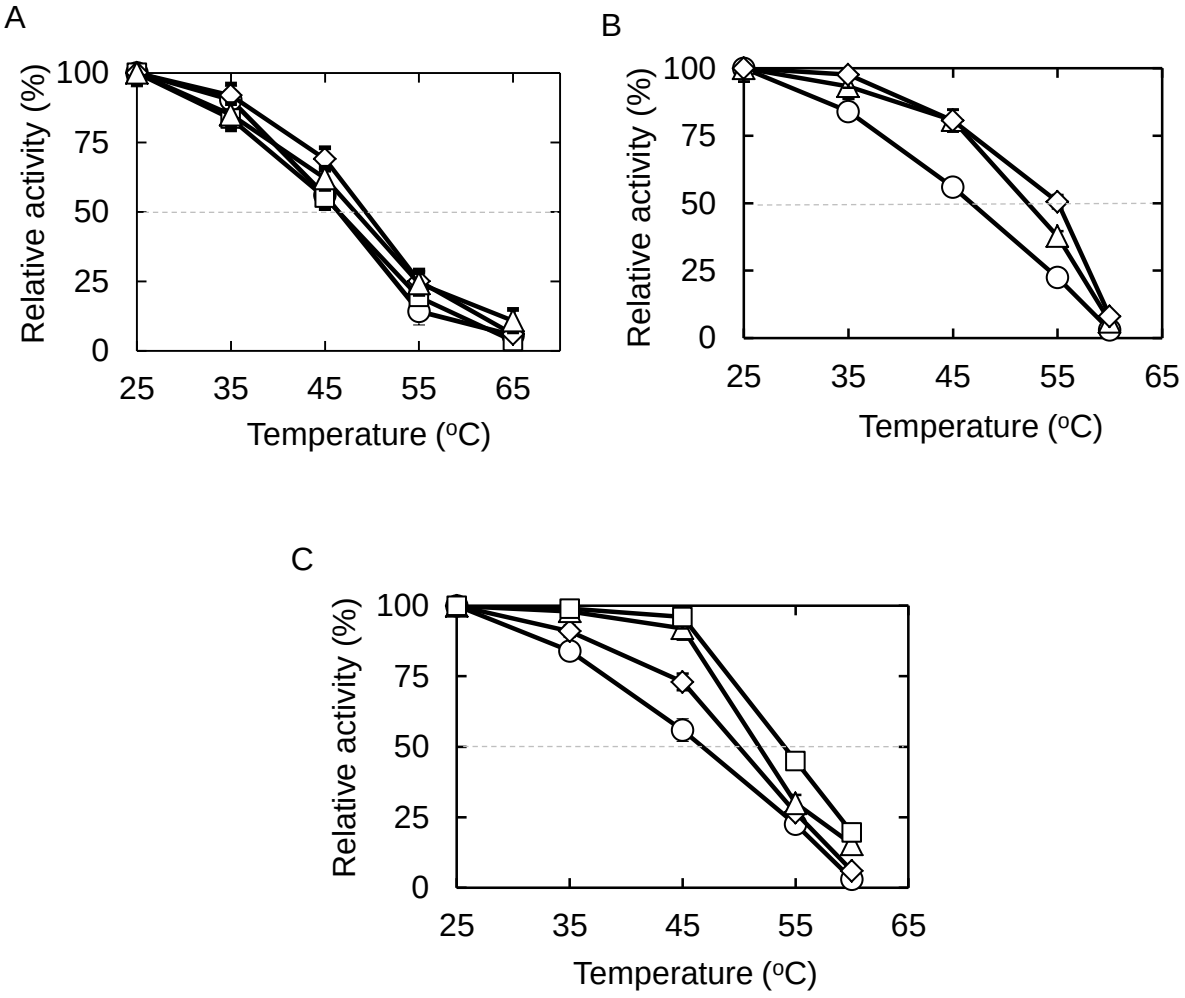

Fig. 6. The thermal stability of modified WBA. WBA was modified by various concentrations of: TNBS (A) mPEG (B), and GA (C)**.** The markers in (A) indicate the concentration of TNBS (mM): 0 (O), 0.5 (□), 5 (Δ), and 10 (◇). The markers in (B) indicate the concentration of mPEG (μM): 0 (O), 1.2 (Δ), and 4.8 (◇). The markers in (C) show the concentration of GA (mM): 0 (O), 1.1 (◇), 4.4 (Δ), and 10.6 (□). The starch hydrolysis activities of the unmodified and modified WBA without heat treatment at 25°C were considered to be 100% and the relative activities at various temperatures were relative to that of at 25°C.

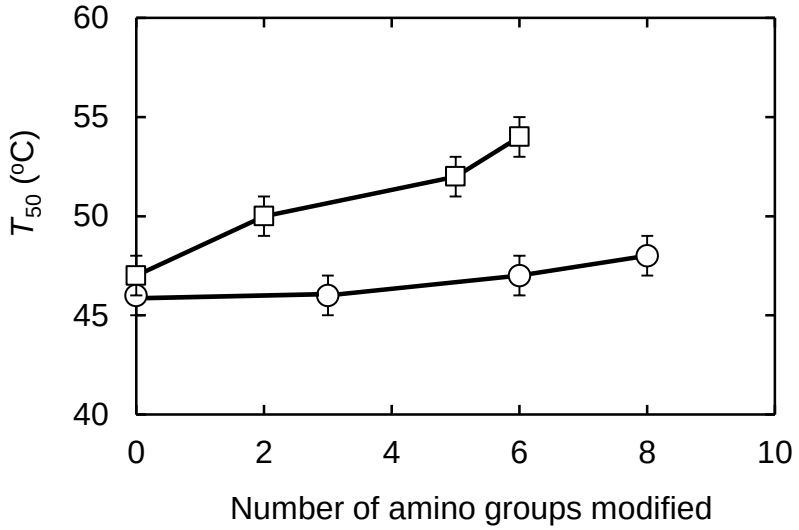

Fig. 7. The relationship between the number of amino groups modified and the T_{50} of WBA. The markers are: O, TNBS and □, GA. The modifications were at 30 min incubation time with increasing concentrations of TNBS and GA. The concentrations of TNBS were 0 (buffer B), 1, 5, and 10 mM while the concentrations of GA were 0, 1, 4, and 11 mM.

Summary

Chapter 1

The kinetic and thermodynamic parameters of WBA were characterized and various additives were evaluated for enhancing its activity and thermostability. The activity of WBA was examined by neocuproine method using soluble starch as a substrate. The K_m and k_{cat} were determined to be 1.0 ± 0.1 % (w/v) and 94 ± 3 s^{-1}, respectively, at pH 5.4 and at 25^oC. The T_{opt} for WBA activity was 55^oC, and the T_{50} was 50 ± 1^oC. Modifications of the solvent with 182 mM glycine and 0.18% (w/v) gelatin increased the T_{50} by 6 and 4^oC, respectively. Glycerol, ethylene glycol, and DMF have also slightly improved the thermal stability possibly through weakening the water structure and decreasing the water shell around the WBA protein. Ethanol enhanced the activity of WBA by up to 24% at 25^oC, probably by inducing favorable conformation for the active site or changing the substrate structure by weakening the hydrogen bonding. Its half-life in the inactivation at 55^oC was improved from 23 to 48 min by 182 mM glycine. The thermodynamic parameters indicate that WBA is thermo-labile and sufficient stabilization was achieved through solvent modification with additives and that the heat inactivation of WBA is entropy-driven. It is suggested that WBA can be applied more widely in starch-saccharification industries with employing suitable additives.

Chapter 2

Inhibition of WBA by glucose and maltose was studied by kinetics and thermodynamics. The inhibitory effects of fructose, difructose, sucrose, trehalose, cellobiose, acarbose, and 1-deoxynojirimycin on WBA were also evaluated. The half maximal inhibitory concentrations (IC_{50}) of acarbose, maltose, and glucose were 0.06 ± 0.01 M, 0.22 ± 0.09 M, and 1.41 ± 0.17 M, respectively. The K_i and the thermodynamic parameters such as $\Delta G^{o\ddagger}$, $\Delta H^{o\ddagger}$, and $\Delta S^{o\ddagger}$ of the dissociations of the WBA-glucose and WBA-maltose complexes were temperature- and pH-dependent. The dissociations were endothermic and enthalpy-driven. Both glucose and maltose behaved as competitive inhibitors at pH 3.0 and 5.4, at 25^{o}C with respective K_i values of 0.33 ± 0.02 M and 0.12 ± 0.03 M, respectively. In contrast, both sugars exhibited uncompetitive inhibition at pH 9.0, at 25^{o}C with K_i values of 0.21 ± 0.03 M for glucose and 0.11 ± 0.04 M for maltose. The pH-dependence of the inhibition type and K_i values indicate that the ionizable groups of WBA influence the interaction with these carbohydrates. This evidence enables us to consider temperature and pH in the WBA-catalyzed hydrolysis of starch to manipulate the inhibition by end-product, maltose, and even by glucose.

Chapter 3

Fluorescence of WBA was quenched by the interaction with maltose or glucose, which are competitive inhibitors of WBA, suggesting that the states of tryptophan and tyrosine residues could be changed by the interaction. The fluorescence emitted by

excitation at 280 nm and 295 nm was titrated by changing the concentrations of maltose and glucose. The K_d values of the WBA-maltose and WBA-glucose complexes were determined to be 0.20 ± 0.12 M for maltose and 0.36 ± 0.11 M for glucose at 25^{o}C, pH 5.4. Maltose exhibited additional binding mode at higher concentration with a distinct K_d value (1.5 ± 0.4 M). The K_d values at various temperatures and pHs are in agreement with the K_i values previously reported in Chapter 2. The negative ΔH^o of the WBA association with glucose and maltose indicate that the associations are exothermic. The K_a and ΔG^o values of the maltose and glucose binding to WBA decreased slightly with increasing temperature from 25 to 45^{o}C but not dependent on pH change (pH 3.0, 5.4, and 9.0). Fluorescence of WBA could be used as a probe to examine the inhibitory interaction with the products of starch hydrolysis.

Chapter 4

The amino groups of WBA were modified by TNBS, mPEG, and GA to improve its thermal stability and activity. Modification of WBA by 5 mM TNBS, 5 μM mPEG and 11 mM GA improved its T_{50} from 47 ± 1 to 48 ± 2, 55 ± 2, and 54 ± 2^{o}C, respectively. The catalytic activity of WBA was reduced by 15% and 59% with modification by 5 mM TNBS and 11 mM GA, respectively. The activity of WBA modified by 5 μM mPEG was enhanced by 39% at 25^{o}C. Therefore, the thermal stability of WBA was significantly improved by modification with mPEG, GA and slightly by TNBS, and its catalytic activity was enhanced by mPEG.

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Acknowledgements

This study was carried out in the Laboratory of Enzyme Chemistry, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, from 2010-2014.

I am very grateful to my supervisor Dr. Kuniyo Inouye, Professor Emeritus of Kyoto University for accepting me, for his highly credible, and excellent guidance in my study.

I would like to thank Mr. Kenji Kojima, Assistant Professor of Kyoto University for his very polite guidance, and dedication to help.

I am thankful to Professor Kiyoshi Yasukawa for his guidance, help, and encouragements. I thank Dr. Teisuke Takita for his scientific contribution, and encouragement.

I would like to express my heartfelt thanks to the Laboratory of Enzyme Chemistry staff, Food Science and Biotechnology, and Foreign Student Divisions of Kyoto University for maintaining very convenient academic environment.

I am grateful to Japanese Government for providing me MEXT (Monbukagakusho) scholarship without which, I could not be able to accomplish my study.

I appreciate the patience and support of my wife, Tsehay Edossa, and my children Yeron and Kena. I am so grateful to my parents, brothers, and sisters for their encouragement. I thank the Almighty God for everything.

March 2014

BEDADA TADESSA DABA

List of Publications

Original Papers

1. Daba, T., Kojima, K., and Inouye, K. (2012) Characterization and solvent engineering of wheat β-amylase for enhancing its activity and stability. *Enzyme Microb. Technol.* **51**, 245-251
2. Daba, T., Kojima, K., and Inouye, K. (2013) Kinetic and thermodynamic analysis of the inhibitory effects of maltose, glucose, and related carbohydrates on wheat β-amylase. *Enzyme Microb. Technol*. **52**, 251-257
3. Daba, T., Kojima, K., and Inouye, K. (2013) Interaction of wheat β-amylase with maltose and glucose as examined by fluorescence. *J. Biochem.* **154,** 85-92
4. Daba, T., Kojima, K., and Inouye, K. (2013) Chemical modification of wheat β-amylase by trinitrobenzenesulfonic acid, methoxypolyethylene glycol, and glutaraldehyde to improve its thermal stability and activity. *Enzyme Microb. Technol*. **53**, 420-426